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AN ELECTROANALYTICAL TOOLBOX FOR BIOAPPLICATIONS

Doctoral Dissertation

Sanna Carlsson

Helsinki University of Technology
Faculty of Chemistry and Materials Sciences
Department of Chemistry

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Abstract

This thesis describes the utilization of electrochemical methods for studying polyion-surfactant interactions, drug transfer through biomimetic phospholipid membranes and lateral charge transfer in DNA. Different aspects of oligonucleotide drug development and the corresponding delivery problem are considered and thus, the literature review in the beginning of the thesis is divided into four parts, giving an overview of the research field.

The problem of oligonucleotide delivery has been approached using a surface-active compound as a carrier for the oligonucleotide. The complexation of the oligonucleotide with the surfactant shields most of the oligonucleotide's negative charges, thus making its incorporation into the lipophilic cell membrane and transfer through it more probable.

Thermodynamics and kinetics of surfactant-oligonucleotide binding have been measured using advanced electrochemical methods based on the electrified liquid-liquid interface. A micropipette-based technique provided information on the stability and energetics of surfactant-oligonucleotide complexes, as well as the degree of binding. The kinetics of the surfactant-oligonucleotide interaction has been approached with a hydrodynamic technique enabling the control of the residence time of surfactant and oligonucleotide.

Membrane activity is the most important property of the oligonucleotide-carrier complex. Carrier complexes should penetrate the biological phospholipid membrane, then escape the interior of the lipid membrane and transfer to the interior of the cell. Membrane activity of the surfactant-oligonucleotide complexes has been studied using a biomimetic phospholipid monolayer immobilised at an electrified liquid-liquid interface.

In a related study, charge transfer in DNA immobilized on silicon surface has been studied by scanning electrochemical microscopy (SECM) to get fundamental information on nano-sized immobilized DNA-architectures.

Keywords oligonucleotide, DNA, polyelectrolyte, surfactant, liquid-liquid electrochemistry, SECM, AFM

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Tiivistelmä

Tässä työssä on tutkittu polyioni-surfaktantti vuorovaikutusta, lääkeaineen jakautumista biomimeettisten fosfolipidikalvojen läpi sekä varauksen etenemistä DNA:ssa sähkökemiallisia menetelmiä hyödyntäen. Oligonukleotidi-lääkeaineiden kehitystä ja niihin liittyvää kuljetusongelmaa on tarkasteltu monista näkökulmista ja tämän vuoksi työn alussa esitetty kirjallisuuskatsaus on jaettu neljään osaan antaen yleiskuvan koko tutkimuskentästä.

Oligonukleotidien kuljetusongelmaa on lähestytty käyttämällä pinta-aktiivista yhdistettä oligonukleotidin kantajana. Kun oligonukleotidi kompleksoidaan surfaktantin kanssa, suurin osa oligonukleotidin negatiivisista varauksista suojautuu ja oligonukleotidi-surfaktantti -kompleksi läpäisee todennäköisemmin lipofiilisen solumembraanin ja kulkeutuu sen lävitse.

Surfaktantin ja oligonukleotidin sitoutumisen termodynamiikkaa ja kinetiikkaa on mitattu sähkökemiallisesti neste-neste -rajapinnalla. Mikropipetti-tekniikka tuotti informaatiota surfaktantti-oligonukelotidi -kompleksien stabiilisuudesta, termodynamiikasta sekä surfaktantin sitoutumisasteesta. Surfaktantti-oligonukleotidi -vuorovaikutuksen kinetiikkaa on lähestytty kanavavirtaustekniikalla, joka mahdollistaa surfaktantin ja oligonukleotidin vuorovaikutusajan kontrollin.

Membraaniaktiivisuus on oligonukleotidi-kantaja -kompleksin tärkein ominaisuus. Kantajakompleksin tulisi läpäistä biologinen fosfolipidimembraani, tämän jälkeen karata lipidimembraanin sisältä ja kulkea solun sisälle. Surfaktantti-oligonukleotidi -kompleksin membraaniaktiivisuutta on tutkittu käyttämällä biomimeettistä fosfolipidi-monokerrosta, joka on immobilisoitu sähköistetylle neste-neste -rajapinnalle.

Tämän lisäksi on tutkittu varauksen etenemistä piipinnalle immobilisoidussa DNA:ssa käyttäen sähkökemiallista pyyhkäisymikroskooppia (scanning electrochemical microscope, SECM) tarkoituksena saada perustavan laatuista tietoa immobilisoiduista DNA-arkkitehtuureista.

Asiasanat oligonukleotidi, DNA, polyelektrolyytti, surfaktantti, neste-neste sähkökemia, SECM, AFM

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Preface

The experimental part of this thesis has been carried out at the Laboratory of Physical Chemistry and Electrochemistry, Helsinki University of Technology during the period from January 2002 until May 2007, excluding the 7 months in 2003 when I visited the University of Newcastle upon Tyne and my maternity leaves in 2004-2005 and 2006-2007. I sincerely thank the graduate school ESPOM, the Finnish graduate school system as a whole and the Finnish Funding Agency for Technology and Innovation (Tekes) for financial support which enabled me to complete this work. I thank my supervisor Professor Kyösti Kontturi and Docent Anna-Kaisa Kontturi for giving me the possibility to work as a member of the Electrochemistry group and introducing me to the world of oligonucleotides.

During the course of this work, I have been given support which has helped me over the obstacles. My deepest thanks go to Dr. Bernadette Quinn, who has acted as my unofficial instructor, motivator and friend, all at the same time. Without her encouragement this work might have been buried under my other ongoing duties. I also thank her for proofreading this manuscript. I thank my research colleagues at the Laboratory of Physical Chemistry and Electrochemistry: Annika for her help with the experiments in the beginning of my postgraduate studies and proofreading this thesis; Peter for many good ideas and discussions about the modeling stuff; Sarah, my very first tutor in the micropipette stuff and all my other great research fellows: Robert, Tanja, Timo, Toffe, Lasse, Chris, Marja, Päivi, Marjukka, Kirsi and Mari and the permanent staff: Gunilla, Mirkku, Marjut and Margit. I also greatly value the time I spent at the University of Newcastle upon Tyne. I thank my local supervisor Dr. Benjamin Horrocks, whose excitement over the results encouraged me to keep going. Lars, Samson, Andy, Romain and David, I really enjoyed working with you.

As a child, I used to tire my parents with lots of questions about the world around me. The same curiosity has driven me through this work and has made me enjoy the little but important details of new knowledge. I would be the same curious person even without this work and I greatly value the fact that my family approves of me as a daughter, sister, wife or mother also without any fine titles. Thank you for your lasting support. My husband Thomas and sons Mathias, Oliver and Tommy, mummy does not have to write during the nights anymore. I love you.

Sanna Carlsson

Espoo, September 23rd, 2009

Table of Contents

List of Publications

I) Hakkarainen, S., Gilbert, S., Kontturi, A.-K., Kontturi, K., Amperometric method for determining the degree of complexation of polyelectrolytes with cationic surfactants, *J. Colloid Interf. Sci.* **272** (2004) 404-410.

II) Hakkarainen, S., Kontturi, A.-K., Kontturi, K., Urtti, A., Electrochemical and calorimetric study of oligonucleotide complexation with cetylpyridinium chloride, *Eur. J. Pharm. Sci*. **23** (2004) 371-377.

III) Carlsson, S., Liljeroth, P., Kontturi, K., Novel Channel Flow Configuration for Studying the Kinetics of Surfactant-Polyelectrolyte Binding, *Anal. Chem*. **77** (2005) 6895-6901.

IV) Carlsson, S., Kontturi, A.-K., Kontturi, K., Improving membrane activity of oligonucleotides by cetylpyridinium chloride: An electrochemical study, *Eur. J. Pharm. Sci.* **29** (2006) 451-459.

V) Lie, L.H., Mirkin, M. V., Hakkarainen, S., Houlton, A., Horrocks, B. R., Lateral charge transport in metal complex-DNA films synthesized on Si(111) surfaces, *J. Electroanal. Chem*. **603** (2007) 67-80.

Author's Contribution

Publications I-IV deal with oligonucleotide-surfactant interaction and the membrane activity of oligonucleotide-surfactant complexes. Sanna Carlsson planned and carried out all the experimental work in Publications II, III and IV and most of the work in Publication I. Publication I was planned in collaboration with Dr. Sarah Gilbert. Carlsson was responsible for all data-analysis included in Publications I, II and IV. The theoretical calculations and analysis were shared (25:75) between Sanna Carlsson and Docent Peter Liljeroth in Publication III. Carlsson was actively involved in the interpretation of experimental data, the development of the mathematical models and the preparation and writing of all these manuscripts.

Publication V deals with SECM measurements of charge transport in DNA-based monolayers on Si electrodes. Sanna Carlsson's contribution was the probe microscopic study, mainly AFM, of the monolayers. The experimental work was shared roughly 50:50 between Lars Lie and Sanna Carlsson; the other authors provided theoretical calculations & input in directing the work.

Professor Kyösti Kontturi
Espoo, April 16th, 2009

List of Abbreviations

2OMe AON	2'-*o*-methyl phosphorothioate antisense oligonucleotides
A	adenine
AC	alternating current
AFM	atomic force microscopy
AIDS	acquired immune deficiency syndrome
AON	antisense oligonucleotide
C	cytosine
CAC	critical aggregation concentration
$CE_{1/2}$	aqueous/organic counter electrode
CMC	critical micelle concentration
CMV	cytomegalovirus
CPC	cetylpyridinium chloride
CTAB	cetyltrimethylammonium bromide
DCE	1,2-dichloroethane
DNA	deoxyribonucleic acid
DOPE	dioleylphosphatidylethanolamine
DoTAB	dodecyltrimethylammonium bromide
DOTAP	N-(1-(2,3-dioleoyloxy)propyl)-N,N,N-trimethylammonium methylsulfate
DOTMA	N-(2,3-(dioleyloxy)propyl)-N,N,N-trimethyl ammonium chloride
DPC	dodecylpyridinium chloride
dsDNA	double stranded deoxyribonucleic acid
FDA	US Food and Drug Administration
G	guanine
HIV	human immunodeficiency virus
IT	ion transfer
ITIES	interface between two immiscible electrolyte solutions
LNA	locked nucleic acid oligodeoxynucleotides
MP	metylphosphonate
mRNA	messenger ribonucleic acid

NaPSS	sodium polystyrene sulphonate
NB	nitrobenzene
NMR	nuclear magnetic resonance
NPOE	2-nitrophenyl octylether
O	oxidized form of redox mediator
ON	oligonucleotide
PG-DNA	single stranded DNA prior to deblocking of the phosphate and nucleobase protecting groups
PNA	peptide nucleic acid
p-Si(111)	positively doped Si(111)
PSS	polystyrene sulphonate polyanion
PVC	polyvinylchloride
R	reduced form of redox mediator
$RE_{1/2}$	aqueous/organic reference electrode
RNA	ribonucleic acid
RNase-H	ribonuclease, which cleaves the 3'-O-P-bond of RNA in a DNA/RNA duplex to produce 3'-hydroxyl and 5'-phosphate terminated product
SECM	scanning electrochemical microscopy
Si(111)	a specific set of atomic planes in silicon's diamond lattice structure
ssDNA	single stranded deoxyribonucleic acid
T	thymine
TTAB	tetradecyltrimethylammonium bromide
UME	micro-sized metal electrode

List of Symbols

a_i^x	activity of the ion i in a phase x
α	charge transfer coefficient
b	distance between neighbouring charges
C	interfacial capasitance
c_i^x	concentration of the ion i in a phase x
C_i^*	bulk concentration of the ion i
D_i	diffusion coefficient of the ion i
D_{surf}	diffusion coefficient for a lateral charge transport
$\Delta_o^w\phi$	Galvani potential difference
$\Delta_O^W\phi_i^0$	standard transfer potential of the ion i
$\Delta_O^W\phi_{1/2}$	half-wave potential
$\Delta G_{i,trans.}^{0,w\to o}$	standard Gibbs energy of transfer
ε	elementary charge
$\varepsilon\varepsilon_0$	permittivity of aqueous solution
F	Faraday constant
i	current
i_L	limiting current
k	Boltzmann constant
K	intrinsic equilibrium constant
k_b^0	backward rate constant
k_f^0	forward rate constant
Kw	co-operative equilibrium constant
P	partition coefficient of the drug
Q	thermodynamic charge
R	universal gas constant
r	radius of micro-ITIES
T	absolute temperature

w	co-operativity constant
x	charge density parameter
z_i	charge of the ion i

1. Introduction

The concept of antisense oligonucleotide strategy has been known for over two decades. The strategy can be simplified as a method for silencing a bad gene by a short fragment of a single stranded DNA. The simplicity of modifying the base sequence of the oligonucleotide to target specific messenger-RNA makes the strategy superior to existing medicinal treatments.

An antisense oligonucleotide has to transfer into the interior of the biological cell to be able to function as a gene silencing agent. The delivery problem of the oligonucleotide has been considered concurrently with oligonucleotide drug development, and it is the main objective of this thesis. The problem arises from the large size and hydrophilicity of negatively charged oligonucleotides, which make penetration through the lipophilic cell membrane difficult. Proposed solutions to this problem have included the use of carriers that can transfer through the cell membrane and deliver oligonucleotide as a cargo. Carriers used include liposomes, polymer compositions, viruses, cyclodextrin, dendrimers and nanoparticles. Toxicity and immunocompatibility of the carriers have hampered development. In this thesis, the possibility of using surface-active agents as oligonucleotide carriers is considered.

Electrochemical methodology has been applied to biochemical and pharmaceutical studies during the last decade. The dependence of the pharmaceutical activity of drugs and their standard Gibbs free energy has been considered in many studies and the development of novel experimental techniques has taken enormous steps. It is now possible to follow drug partitioning through the lipid monolayer immobilized at the interface of aqueous phase and organic solvent using electrochemical techniques. The electrified water-organic interface suits well for the oligonucleotide-surfactant study as it enables detection of these highly charged species using above mentioned advanced techniques. The liquid-liquid interface can also be used as an analytical method for detecting the concentration of the unbound surfactant in the aqueous phase. Techniques and methods used in this work include chronoamperometry at the supported water-organic interface using micropipette and channel flow configurations; AC-voltammetry at interfaces modified using Langmuir-Blodgett methodology; scanning electrochemical microscopy (SECM) as well as atomic force microscopy (AFM). Calorimetry was used to obtain additional information about the energetics of the oligonucleotide-surfactant complexes.

This work has been divided in two parts: the literature section (Chapters 2-5) and the results section (Chapters 6-7).

Chapters 2-5 constitute the literature part of the thesis. Chapter 2 considers the electrochemistry of the liquid-liquid interface and gives a summarized overview of the basis of charge transfer through the interface between mutually immiscible phases. The main emphasis has been placed on the interplay

between pharmaceutics and electrochemistry. Chapter 3 gives an overview of recent developments in antisense oligonucleotide research. This chapter concentrates on the key concepts of the antisense strategy, discusses the reasons for using oligonucleotide modifications and considers the delivery problem and current status of clinical trials. As the main objective of the work was to test the applicability of surface-active compounds as oligonucleotide carriers, Chapter 4 considers the interaction between surfactants and poly- and oligoelectrolytes. The chapter discusses the effect of the polyelectrolyte and surfactant type on the stability of the polyelectrolyte-surfactant complex and the efficiency of the interaction. Chapter 5 briefly introduces the recent research considering charge transfer in DNA.

Chapter 6 summarizes the results of Publications I-V. Publications I-II considered complex formation between the surfactant and oligo-/polyelectrolytes aiming to find a surfactant type, which forms stable complexes with oligonucleotides. In Publication I, the complex formation and applicability of a micropipette-supported liquid-liquid interface as an analysis system were first extensively tested with polystyrene sulphonate in a proof of concept study prior to utilizing oligonucleotides. The method was then applied to study of oligonucleotide-surfactant interaction in Publication II. The subject of Publication III was kinetics of surfactant-polyelectrolyte binding. The matter was studied with an advanced channel flow method using the liquid-liquid interface as an ion-selective electrode. The membrane activity of oligonucleotides and their complexes with surfactant were studied in Publication IV using a Langmuir-Blodgett based method and electrochemical analysis. Publication V considered charge transfer in immobilized DNA. The subject was approached using SECM and AFM methods. Chapter 7 details the conclusions of the thesis.

The subject of the thesis focuses on the interplay between electrochemistry, pharmaceutics and bioanalytics. While knowledge of electrochemistry is essential, analyzing the results requires an interdisciplinary understanding in the above mentioned research fields. This thesis, including the literature part and result section, is responding to that demanding task.

2. Electrochemistry at ITIES

Electrochemistry at the interface between two immiscible electrolyte solutions (ITIES) differs from conventional electrochemical experiments in that the reaction under study occurs at a liquid-liquid interface rather than at a typical electrode/electrolyte interface. In addition, charge transfer across the immiscible interface is not limited to the study of electron transfer reactions. Ions transferring from one phase to the other can also be elegantly studied using standard electrochemical methodology. Thus, a large number of ion transfer reactions important to biological systems can be studied using liquid-liquid electrochemistry that would be impossible with conventional metal electrodes. The breakthrough for experimental studies at ITIES was the demonstration that the interface between two immiscible solvents is polarisable and that the Galvani potential difference between two phases, defined as

$$\Delta_o^w \phi = \Delta_o^w \phi_i^0 + \frac{RT}{z_i F} \ln \frac{a_i^o}{a_i^w} \quad (1)$$

could be used as a driving force for charge transfer reactions from one phase to another. [1-3] In equation 1, $\Delta_o^w \phi_i^0$ refers to the standard transfer potential of ion *i*, z_i is the charge of the ion *i* and a_i^x is the activity of ion *i* in a phase *x*. Superscripts *o* and *w* refer to the organic and aqueous phase, respectively. ITIES electrochemistry was further developed to its current state during the 1970's and 1980's.[4-8]

The main criterion for ITIES used in modern electrochemical measurements is that it should be polarisable i.e. there should be a $\Delta_o^w \phi$-range where ion transfer from one phase to the other is negligible. This criterion limits the choice of the organic solvent and supporting electrolytes that can be used. The organic solvent should have a minimal miscibility with the aqueous phase, while its dielectric constant should be sufficiently high so that the supporting electrolyte salt is dissociated rather than ion-paired. Typical choices for the organic solvent are nitrobenzene (NB), 1,2-dichloroethane (DCE) and 2-nitrophenyl octylether (NPOE). The latter is considered an environmentally friendly option when compared to NB or DCE. The aqueous/organic supporting electrolyte has to be sufficiently hydrophilic/hydrophobic for the interface to be polarisable.

The supporting electrolyte ions have two roles in electrochemical experiments: 1) they carry charge, which is beneficial as migration can be neglected when solving the corresponding transport problem for ion transfer across the interface; 2) they set the limits for the applicable, polarisable $\Delta_o^w \phi$-range, which is called the potential window. The potential window increases with increasing hydrophobicity of the

organic base electrolyte ions for a given aqueous base electrolyte. The study of ion transfer reactions is limited to those that occur within the potential window.

2.1. Ion transfer

Electrochemistry at ITIES is most commonly used for studying ion transfer (IT) reactions. IT is defined as the transfer of an ion from one phase to the other and the reaction scheme given here is for the transfer of an aqueous cation to the organic phase:

$$X^+(aq) \leftrightarrow X^+(org) \tag{2}$$

IT of an ion *i* can be characterized by its standard transfer potential, $\Delta_o^w \phi_i^0$, which is a measure of the ion's relative hydrophilicity/hydrophobicity and is defined as

$$\Delta_o^w \phi_i^0 = \frac{\Delta G_{i,trans}^{0,w\to o}}{z_i F} \tag{3}$$

where $\Delta G_{i,trans}^{0,w\to o}$ is the standard Gibbs energy of transfer. For example, $\Delta_o^w \phi_i^0$ shifts negative when the hydrophobicity of an organic phase cation is increased implying that it will remain in the organic phase unless a very negative potential difference is applied to the interface. The situation is vice-versa for an organic phase anion. The potential at which an ion transfers is a useful intuitive guide to its relative lipophilicity.

The kinetics of ion transfer across the interface is generally considered to be very rapid and the rate is nowadays assumed to be diffusion limited. However, the subject of ion transfer kinetics was rather controversial during the development of modern liquid-liquid electrochemistry, due mainly to uncertainty of the interfacial molecular structure. Many interpretations for the interfacial structure have been put forward in the past.[9] The first proposed model approximated the interfacial region as a compact layer of oriented solvent molecules separating two diffuse layers.[10,11] The theoretical description of the interfacial capacitance, defined as

$$C = \frac{\partial Q}{\partial \Delta_o^w \phi} \tag{4}$$

was also derived, and was an extension of the conventional Gouy-Chapman model used to describe the metal/electrolyte interface.[12] The parameter Q in equation (4) is the thermodynamic charge. Other significant models for the interfacial structure include a mixed solvent layer[13], which is generally the most accepted model. It has been experimentally proven that the potential drop across this kind of interfacial layer is negligible.[14] Despite the uncertainty concerning the interfacial structure, charge transfer kinetics have generally been treated with the Butler-Volmer equation, which is written as

$$\frac{i}{F} = k_f^0 c_i^w exp\left[\frac{\alpha F \Delta_o^w \phi}{RT}\right] - k_b^0 c_i^w exp\left[\frac{-(1-\alpha) F \Delta_o^w \phi}{RT}\right] \quad (5)$$

where k_f^0 and k_b^0 refer to forward and backward rate constants, respectively; and α is the charge transfer coefficient. The compatibility of Equation 5 with experimental results has been satisfactory.[9]

Scaling down the size of the interface to the micro-scale (i.e. smaller than the typical thickness of the diffusion layer) is advantageous. The lowered ohmic drop, enhanced mass transfer and the possibility of performing experiments in micro-environments such as living cells are direct consequences of scaling down. Mass transfer of the transferring ion is controlled by spherical diffusion to the micro interface. Thus, the current response to a potential step at long times approaches steady state, in contrast to that at a conventionally sized interface where it approaches zero. A time-independent current response is obviously very desirable in electrochemical sensing applications. The limiting current, i_L, obtained after application of a potential step to the diffusion limited region can be written as[15]

$$i_L = 4 z_i F D_i C_i^* r \quad (6)$$

where z_i is the charge, D_i is the diffusion coefficient, C_i^* is the bulk concentration of the ion i and r is the radius of the interface. Micro-ITIES can be created by either supporting the interface at the end of a micropipette[16] or at a microhole photoablated in a thin polymer film[17]. Micro-ITIES has been successfully applied for sensing applications such as amperometric ion-selective sensors.[15,18]

Micro-ITIES has been further scaled down to nanoscale.[19] This further reduces the ohmic drop and enables the measurement of drastically faster ion transfer kinetics. As the rate of ion transfer is determined by both mass transfer and the kinetics of ion transfer across the interface, the significantly enhanced rate of mass transfer at nano-ITIES makes it comparable or even faster than the interfacial kinetics and thus enables the kinetics to be measured. Nano-ITIES also extends the choice of organic solvent/base electrolyte systems that can used beyond those used at conventionally sized interfaces. The very low

currents measured make the ohmic drop across the interface negligible enabling measurements in highly resistive solvents.[20]

Another way to attain steady-state in liquid-liquid electrochemical measurements without having to decrease the interfacial size to the micron range is to use hydrodynamic electrochemical techniques, where convection to the interface is forced.[21] This situation can be achieved either by moving the electrode itself or forcing the solution to flow past a stationary electrode. Advantages of applying these methods to ITIES are the same as for conventional electrode/electrolyte hydrodynamic systems:[22] steady state is attained quickly and measurements can be done with high precision, as the mass transfer is enhanced compared to diffusion as the only mechanism of mass transfer. Time enters the corresponding transport problem either as the electrode rotation rate or as the velocity of the solution with respect to the electrode. Hydrodynamic electrochemical methods utilized at ITIES include the electrolyte dropping electrode,[23] analogous to the dropping mercury electrode; the rotating diffusion cell,[24] analogous to the rotating disk electrode, the wall-jet setup [25] and the channel-flow cell.[26] This area of electrochemistry at ITIES is still relatively new and few studies have been published. Future applications may include amperometric sensors.

2.2. Pharmaceutical viewpoint to electrochemical methods

Electrochemical studies at ITIES can be directed to numerous applications such as amperometric ion sensors, Marangoni pumps, electro-assisted solvent extraction, thermoelectricity, electrocatalysis, solar energy conversion and pharmaceutics.[27] In this work, the focus is on the pharmaceutical viewpoint.

It was long believed that drugs partition through the bio-membrane either in neutral form or as ion pairs. This belief was dispelled when it was shown that drug ions can diffuse passively through the phospholipid membrane.[27] One of the pioneering electrochemical studies concerning pharmacokinetics showed that electrochemical measurements could be used to determine the lipophilicity of ionized drugs at ITIES.[28] It was demonstrated that the drug partition coefficient could be evaluated using the following equation:

$$\ln(P_i^0) = -\frac{z_i F}{RT}\Delta_o^w \phi_{\mathrm{i}}^{0} \tag{7}$$

where the standard ion transfer potential, $\Delta_o^w \phi_{\mathrm{i}}^{0}$, can be written for diffusion-limited ion transfer as

$$\Delta_o^w \phi_{\mathrm{i}}^{0} = \Delta_o^w \phi_{1/2} - \frac{RT}{2zF}\ln\frac{D_i^w}{D_i^o} \tag{8}$$

$\Delta_o^w \phi_{1/2}$ refers to the half-wave potential and is experimentally determined from simple electrochemical methods such as cyclic voltammetry. In cyclic voltammetry, the potential is swept linearly and the current response is recorded. The resulting $\Delta_o^w \phi$ – current response depends on the corresponding mass transport of the ion to the interface.

Many electrochemical studies have focused on the voltammetric response of biologically relevant compounds. Compounds that have been studied include choline and acetylcholine,[29] which have signaling roles in neurotransmission; procaine, tetracaine, cocaine etc.[30,31], which are local anesthetic drugs; calcium antagonists;[32] aromatic amines[33], dipeptides[34], opioids[35], which are used for pain relief and metabolites[35,] which are intermediate products of metabolism. Arai et al. made a comparison between the pharmacological activity of drugs and their $\Delta G_{i,trans}^{0,w\to o}$ and found that especially families of hypnotic, anesthetic, cholinergic, and adrenergic drugs showed a strong correlation between the drug lipophilicity ($\Delta G_{i,trans}^{0,w\to o}$) and biological activity.[36] The pH-dependence of drug transfer has also been studied.[37,38] The results predicted which ionic form of the drug will transfer across the interface under given conditions. The use of electroanalytical techniques at the liquid-liquid interface has recently been intensively studied for the detection of negatively charged heparin, which is a human serum component and is also widely used as an anticoagulant in medical treatments.[39-42] Heparin adsorption at the interface was found to be facilitated by the presence of hydrophobic ammonium cations in the organic phase.

Drug partitioning studies have been extended to include systems where lipids, typically phosphatidylcholines, were adsorbed at ITIES, thus creating a lipid monolayer mimicking half of the biological lipid bilayer. Using a monolayer instead of conventional biomimetic bilayers is advantageous as the potential distribution across the monolayer can be controlled. Early studies showed that the rate of ion transfer across ITIES for most ions decreased significantly upon lipid adsorption.[43] However, for some systems, the rate of ion transfer was noted to increase.[44,45] This discrepancy was explained taking double layer effects into account: i.e., the interfacial potential profile changed due to the presence of zwitterionic phosphatidylcholines.

Monolayers formed by the simple adsorption of lipids to the interface are believed to remain in the liquid-expanded state and studies show possible solvent incorporation into the monolayer region.[47] Due to a desire to control the exact state of the monolayer, the Langmuir method was introduced for controlling the surface pressure of the adsorbed monolayer.[46-48] The Langmuir method was combined with electrochemical control of the potential across the interface. However, this experimental arrangement suffered from complications due to the large interfacial area and the phospholipid monolayer dissolving into the organic phase.

The next advance in drug transfer studies came with the introduction of the Langmuir-Blodgett method to transfer the lipid monolayer formed at the air-water interface to ITIES.[49] Here the lipid monolayer is compressed to the desired surface pressure at the air-water interface and then transferred to the surface of an electrochemical cell suitable for liquid-liquid studies by simply dipping the cell through the monolayer as shown in Figure 1. Transfer of the monolayer to the interface was made possible by using an immobilized organic phase. The Langmuir-Blodgett method enabled full control of the state of the monolayer formed at the interface and for the first time, ion transfer across the interface modified by the monolayer could be investigated as a function of the state of the monolayer, i.e., whether it was in a liquid-expanded, liquid-condensed or collapsed state. This experimental approach has been used for studying partitioning and adsorption of drugs to the lipid monolayer using AC-voltammetry and AC-impedance: electrochemical techniques more sensitive than simple cyclic voltammetry. A surface pressure dependence, i.e. a dependence on the state of the monolayer, was demonstrated for the rate of drug transfer. [50]

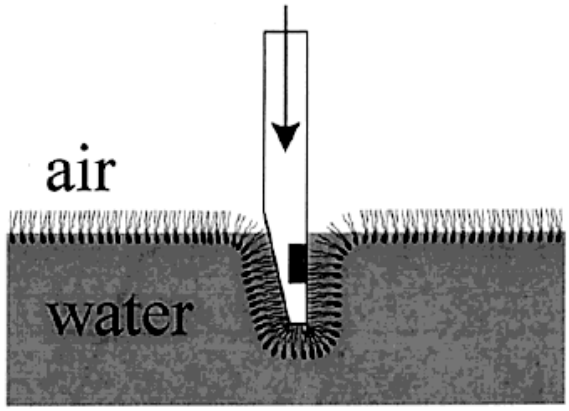

Figure 1. Schematic illustration of the Langmuir-Blodgett method applied to liquid-liquid interfaces. The electrochemical half-cell is dipped through the lipid monolayer formed at the air-water interface. The potential difference between the water and the immobilized organic phase (shown as the dark grey compartment of the electrochemical cell) could then be fully controlled. Reproduced in part with permission from reference [50]. Copyright 2001 American Chemical Society.

Another way to study biological systems using electrochemical techniques at ITIES is to utilize ultramicroelectrodes (UME) or scanning electrochemical microscope (SECM) techniques in the proximity of interesting biological surfaces. SECM has been widely used during the last ten years and a review has

been published on the subject.[51] The traditional SECM tip is a micro-sized metal electrode (UME) surrounded by a thin, well defined layer of insulating material, typically glass. In a typical SECM experiment, the measurement is performed in so-called feedback mode: the SECM tip is immersed in the solution containing a redox mediator, R. The UME is biased at a potential in the diffusion-limited region for the oxidation of R to O:

$$R + ne^- \rightarrow O \qquad (9)$$

The potential is chosen such that the rate of the reaction is limited by the diffusion of R to the tip. If the tip is sufficiently far away from the substrate (surface under study), the current is given by equation (6) similarly to the diffusion limited current at micro-ITIES. When the tip is brought in close proximity to the substrate (within a few tip radii), the tip current response is dependent on the nature of the substrate. If the substrate is conductive, the product O formed at the SECM tip diffuses to the substrate and is reduced back to R, thus increasing the current response at the SECM tip. This additional flux of R at the tip is called positive feedback (see Figure 2). If the substrate is an insulator, it blocks the diffusion of R species to the tip and the current is lower than the limiting current recorded far away from the substrate under study. This response is called negative feedback.

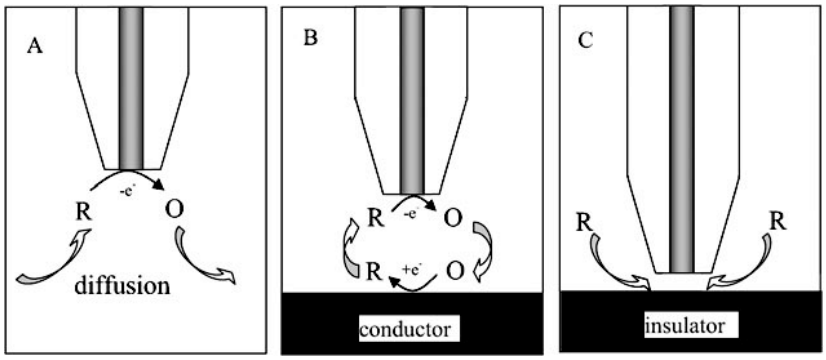

Figure 2. Feedback mode of the SECM operation. (A) The UME tip is far from the substrate. (B) Positive feedback: species R is regenerated at the substrate. (C) Negative feedback: diffusion of R to the tip is hindered by the substrate. [51] Reproduced by permission of the PCCP Owner Societies. http://dx.doi.org/10.1039/b612259k

The metallic SECM probe can be replaced with a micropipette containing a liquid phase (usually organic) and a liquid-liquid interface is then formed upon immersion of the micropipette into the aqueous phase. With this approach, SECM can be extended to study ion partitioning in biological systems.

Pioneering work was done by Amemiya and Bard who measured potassium ion fluxes through gramicidin channels incorporated in bilayer-membranes.[52] In a related study, drug adsorption to monolayer membranes was probed.[53]

As can be readily seen from the preceding section, electrochemistry at ITIES provides a very powerful tool for studying charge transfer and adsorption processes in biological systems. Research in this area is still in its infancy and much knowledge and new studies are needed for techniques to be available to the pharmaceutical community. In this work, electrochemical methodology at ITIES has been utilized for studying different aspects of antisense oligonucleotide delivery through the lipid membrane. Electrochemical techniques were used both for studying complex formation of oligonucleotides with possible future delivery agents and the interaction of such complexes with lipid monolayer membranes. Model compounds were used in the proof of concept studies to minimize expense. SECM together with atomic force microscopy (AFM) was used for studying the nano-sized DNA architectures immobilized on silicon. As the subject area is rather broad, an overview of oligonucleotide delivery and complex formation with the surfactant is given in the following two chapters and the subsequent chapter gives a short overview of DNA-mediated charge transfer. The last chapters summarize the experimental work and results of this thesis.

3. Overview of antisense strategy

There is a high mortality associated with diseases resulting from errors in the genetic code. These diseases vary from cancer to HIV. Traditionally, the symptoms have been treated with many different strategies. Their development has been both expensive and time consuming. The nucleic acid based therapies are novel approaches, which treat the cause rather than the effect of the disease. These therapies include antisense oligonucleotides, ribozymes, RNA interference, aptamers and other type of gene therapies. In this overview, the focus is purely put on the antisense oligonucleotide strategy, whose successful implementation would be of great interest for medicinal companies and research groups since this single strategy can be used to treat patients with very different problems.

The basis of antisense strategy was discovered some decades ago, when Zamecnik and Stephenson found that a short fragment of single stranded DNA inhibited the replication of *Rous Sarcoma* –virus.[54] Such a single stranded fragment of DNA, around twenty nucleotides long, is nowadays called an antisense oligonucleotide (AON) (Figure 3).

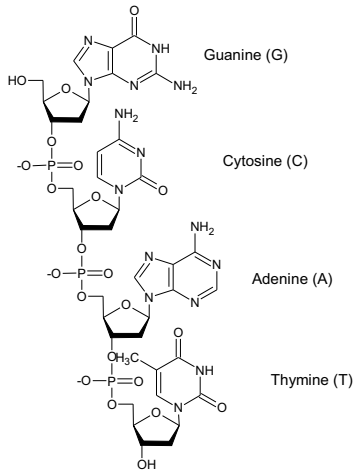

Figure 3. A fragment of oligonucleotide with four different bases: G, C, T or A. One unit of oligonucleotide, a "nucleotide", consists of three parts: 1) a sugar, 2) the base and 3) an internucleotide linkage between two sugars.

The inhibition is achieved with AONs which have a complementary base sequence targeting the sequence of messenger-RNA (mRNA), which delivers the disease-provoking genetic code from the nucleus to the ribosome. The transcription of the genetic code of DNA into synthesized mRNA has been a hot topic during recent years, as Roger D. Kornberg was awarded a Nobel prize for his studies on the

subject.[55,56,57] AON hybridizes to the target mRNA through Watson-Crick base pairing, disables the building of the disease-causing protein (which the mRNA codes for) and activates RNase-H, which hydrolyses the RNA strand of the heteroduplex and thus cleans the improper genetic material away from the cell. While RNase-H activation is preferable, with new AON modifications, the mode of action can also be based on other mechanisms. These include translational arrest of the diseases causing genetic code by steric hindrance of ribosomal activity.[58,59]The idea of the antisense strategy is presented in Figure 4.

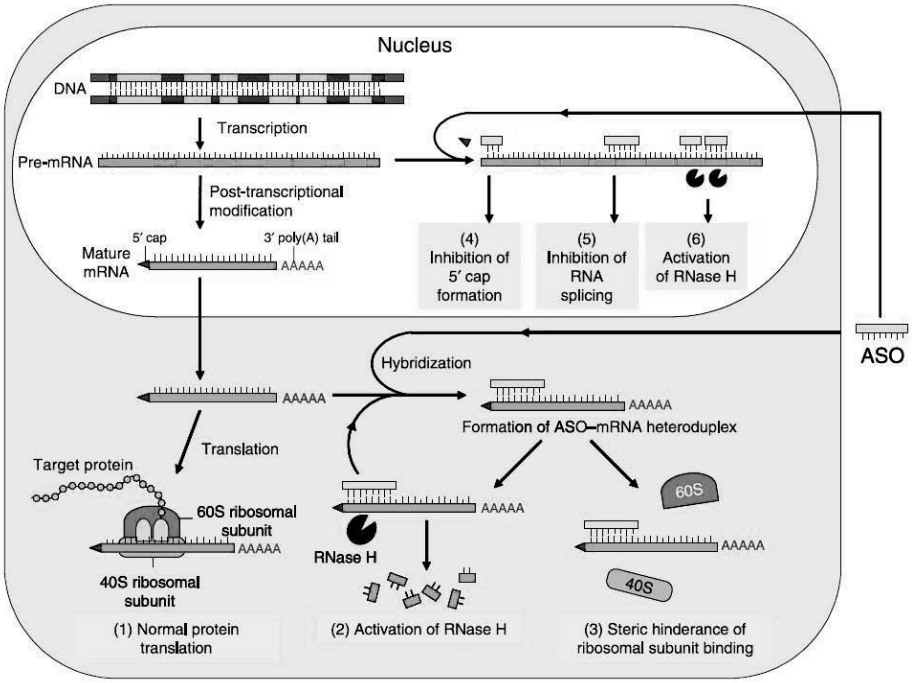

Figure 4. Antisense strategy: The gene is silenced by the oligonucleotide, which hybridizes to the target and prevents protein synthesis. (1) Normal protein translation without AON. (2) – (5) Different routes to silence the gene. The route (2), where the AON activates RNase H is the most desired and effective route for AON to inhibit the protein synthesis. Reproduced with permission from [58]. Copyright 2006 Wiley-Blackwell.

Designing the most effective AON is not simple and requires knowledge of the secondary structure of the target mRNA. The effective AON should target the sites where mRNA is accessible for hybridization,[60] which usually are those located at the terminal end, internal loops, joint sequences, hairpins and bulges of 10 or more consecutive nucleotides.[61] Many algorithms have been tested for the determination of the secondary structure and the most efficient ON sequences.[60,61] Companies such as ISIS Pharmaceuticals

have performed screening and testing by a gene-walking method, where hundreds of AONs are tested against one target mRNA, but this kind of method is inefficient and usually only 2-5 % of screened ONs are found to function as antisense reagents.[62]

3.1. Modifications

The natural form of the oligonucleotide (ON), phosphodiester, has unfavorable properties, the most important of which is its poor serum stability. To improve the serum stability, one of the oligonucleotide's non-bridging oxygens of the phosphate bridge can be replaced with a sulphur, producing a phosphoromonothioate oligodeoxynucleotide.[63] (Figure 5) This modification is often referred to as a first generation oligonucleotide and it meets the requirements of the antisense strategy very well. These requirements include serum stability, binding affinity to the target, aqueous solubility and RNase-H activation.

While the cellular uptake of phosphorothioates is low, it has been reported to be higher than the uptake of other first or second generation AONs.[64] Regrettably, it has been noted that phosphorothioates also perform many non-sequence-specific events leading to the toxicity of the oligonucleotide.[63,65,66] Earlier, it was believed these effects arise from the polyanionic nature of the phosphorothioate.[67] Later studies proved that some specific base motifs produce most of the non-sequence-specific effects. These include the CG base motif, which induces the production of various cytokines and chemokines[65] and the motif of four contiguous guanosine residues, which form a so-called G-tetrad trough the combination of Watson-Crick and Hoogsteen base-pair formation. These G-rich phoshorothioates have a high affinity to heparin binding proteins.[68] If these undesired motifs are necessary for the phosphorothioate to function as was originally intended, some modifications can be made to the backbone of the oligonucleotide to prevent side effects. To inhibit side effects of the CG-motif, cytosines of the CpG-motif can be replaced with 5-methylcytosines, and cytosines and guanosines of the CpG-motif can be replaced with 2'-*O*-methyl cytosines and guanosines, respectively.[63,65] In addition, internucleotide linkage between C and G is often replaced with a neutral methylphosphonate linkage.[69] The G4-motif can be improved by replacing one or more guanosines of the motif by 7-deazaguanosine. Despite disadvantages, the phosphorothioate modification is the most widely performed chemical modification of AONs.

Due to the limitations of phoshorothioates and to further enhance the nuclease resistance and binding affinity, other modifications have also been suggested. Promising results have been obtained with 2'-*O*-methyl phosphorothioate antisense oligodeoxynucleotides (2OMe AON) both in animal studies[70,71] and *in vitro* studies.[72] This modification is less toxic than the phosphorothioate counterpart. Also it has been shown that the 2OMe AON is considerably more efficient in inducing the antisense effect than the comparable phoshorothioate modification. The phoshorothioate linkage seems to be critical for the

function of 2OMe AON, as in a study, 2OMe AONs with phosphodiester linkage performed clearly weaker than the corresponding 2OMe phosphorothioate AONs.[73] The drawback of the 2OMe AON modification is that it does not induce RNase-H activation.[74] It has been further shown that any substitution to the 2'-position of the ribose ring will block the RNase-H activity.[75] In order to combine the advantages of the first generation phosphorothioate AONs and the 2OMe modification, a chimeric ON was introduced. This consisted of the central "gap" region around ten nucleotides, where the non-bridging oxygen was replaced with sulfur (phosphorothioate linkages) and two "wings" in both ends composed of nucleotides carrying the 2OMe modification. The end blocks prevent degradation of the AONs and the central gap residues between 2OMe modified segments were reported to be sufficient for activation of RNase-H.[76]

Methylphosphonates (MP) and peptide nucleic acids (PNA) are electrostatically neutral ON analogs, which belong to the group of most studied AON modifications. MPs are ON analogs, whose internucleotide linkages have been replaced with methylphosphonate. PNAs are RNA analogs with 2-aminoethylglycine backbone. They hybridize well and resist nucleases but posses poor aqueous solubility and cellular delivery. [64,77,78] In addition, PNAs do not activate RNase-H[74].

Locked nucleic acid oligodeoxynucleotides (LNA) are maybe the most promising AON candidates containing a bridging methylene carbon between the 2' and 4' position of the ribose rings. This modification pre-organizes the oligonucleotide backbone, allowing the oligonucleotide to bind special targets and has been shown to hybridize with the target as well as inhibit specific gene expression.[78] While it cannot activate RNase-H alone due to conformational change it affects, a chimeric LNA with a central gap containing phosphodiester/phosphorothioate residues has been shown to have an extraordinary high target affinity and improved RNase-H activation.[74,79] However, *in vivo*-study has shown profound hepatotoxicity for multiple LNA-modified AON sequences.[80]

As the activity of the AON depends on numerous factors such as base sequence, secondary structure of AON and target organ, it is probable that no universally applicable backbone modification exists. Therefore, it is possible that the modification of the AON has to be optimized for each case, if maximum efficiency is desired.

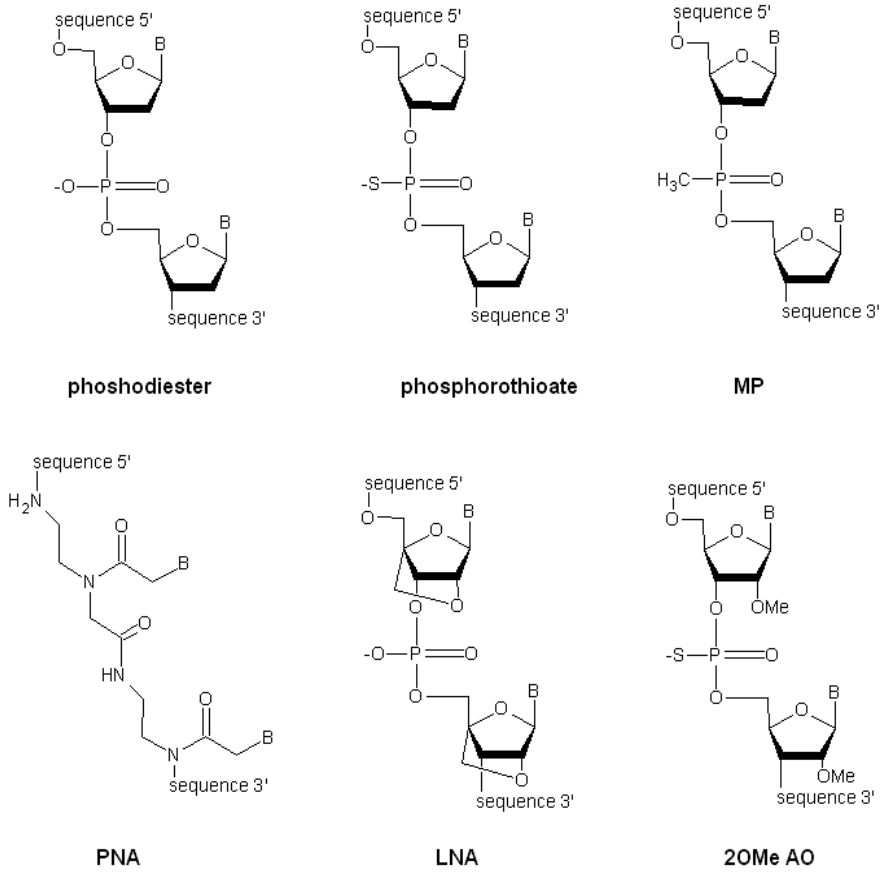

Figure 5. Backbone modifications. B refers to one of the four possible base units.

3.2. Systemic and cellular delivery

The rationale of the antisense strategy is clever. The same strategy can be applied to numerous genetic diseases, cancer and HIV. However, certain critical limitations exist concerning the biodistribution and the cellular delivery of the oligonucleotides. To be successfully transferred to target cells, the oligonucleotide has to fulfill certain requirements: 1) it has to be resistant against serum; 2) it should not have any non-specific interactions with serum components; 3) it has to target specific organs and transfer trough the endothelial barrier of this organ; 4) once inside the desired organ, the oligonucleotide has to penetrate the cell membrane of the target cells and get rid of the possible carrying agent.

The first two conditions were dealt with in the previous chapter and it can be concluded that problems of serum stability and non-specific interactions can largely be solved with the proper backbone

modification. New interaction problems might arise, however, with the use of positively charged carrier agents, which have been shown to interact with albumin and other plasma proteins. The interaction decreases the residence time of the AON-carrier complex in the blood circulation.[81]

The third condition is still largely unsolved and is the most challenging one. In most organs, the structure of endothelia is tight. It has been reported that most of the naked phosphorothioate oligonucleotides[65,73], as well as poly-L-lysine/DNA complexes[82], accumulate rapidly into the liver, which is the organ eliminating toxins from the blood circulation. Intermediate concentrations have been reported to been found in kidney, spleen and bone marrow. Most organs mentioned above have endothelia with large meshes enabling the extravasation of molecules ranging from 0.1 to 1 μm.[83] The specificity to target organs (e.g. brains, tumors) could be achieved by delivery agents, which are specified to target desired destinations.

The biggest effort within AON research has concentrated on resolving the fourth requirement, i.e. to overcome the undesired propensity of the cell membrane to act as a barrier for antisense oligonucleotide activity in most cases. The original function of the cell membrane is to segregate the intracellular and extracellular matter. The extracellular surfaces of most cell membranes are negative under physiological conditions due to negatively charged lipids and proteins and the interior of the membrane is hydrophobic. Both are factors that complicate the partitioning of the negatively charged hydrophilic molecule into the cytoplasm. Partitioning of the oligonucleotide into the nucleus (if necessary) is less problematic in so far as the oligonucleotide has transferred through the outer cell membrane.[84]

Despite the fact that "naked" ONs transfer into the cell only weakly, unmodified ONs have been found in cellular nuclei and cytoplasm without any carrier. The most accepted mechanism of ON transfer is via endocytosis.[85] While the most serious problem for the ON's passive diffusion through the lipophilic cell membrane is the negative charge that phosphodiesters (unmodified form of ON) and phosphorothioates (first generation AON) carry, surprisingly the neutral forms of the modified ONs (methylphosphonates and PNA) have even weaker cellular uptake. This results from the fact that neutral ONs bind weakly to the cell surface proteins and thus exhibit weaker adsorptive endocytosis than anionic ONs.[86]

Penetration through the cell membrane can be improved by carriers. Carriers bind to the oligonucleotide physically or chemically. They have a high affinity to incorporate into the cell membrane and deliver oligonucleotides as a cargo. The most well-known delivery agents are cationic liposomes,[87] and these are commercially available (e.g. Lipofectin®). Liposomes are phospholipid bilayer membranes surrounding an internal aqueous compartment and they act as ON transfection agents either by internalizing the ON inside the aqueous compartment or forming a spontaneous complex with ON driven

by electrostatic interactions. Usually a cationic liposome consists of cationic lipids such as N-(2,3-(dioleyloxy)propyl)-N,N,N-trimethyl ammonium chloride (DOTMA) or N-(1-(2,3-dioleoyloxy)propyl)-N,N,N-trimethylammonium methylsulfate (DOTAP) with neutral unsaturated helper lipids such as dioleylphosphatidylethanolamine (DOPE).[88] It has been shown that the optimal amount of cationic lipid in the oligonucleotide-liposome complex is approximately half of the negative charges of the oligonucleotide.[89] Internalization of the liposome-oligonucleotide complexes has been proposed to occur via the endocytic pathway.[90] Critical factors in the internalization and release processes are the negatively charged proteins at the outer surface of the membrane, which enable interaction with the positive liposomes. Negatively charged lipids at the inner surface of the membrane form neutral lipid pairs with cationic lipids of the liposome at the endocytic stage and act as releasers of the anionic nucleic acid. Many *in vitro* studies have proved the effectiveness of the liposome-mediated oligonucleotide transfection protocol.[91,92] Some concerns have arisen because of the toxicity of high concentrations of lipids, which have to be used for efficient transfection. It has been shown that lipids (especially DOPE) are toxic towards macrophages, which are critical in the control of the immune system.[93] In addition, liposomes have been shown to interact non-specifically with various components of the serum and thus alter the success of transfection.[94]

Another widely studied group of delivery agents are polymer and polyion compositions. Poly-L-lysine conjugated with a membrane receptor-specific transfection agent is the most well known of this group. Such transfection agents employ membrane receptor mediated endocytosis for the uptake in to the cell and include asialoorosomucoid (which is a galactose-terminal glycoprotein),[95] transferrin[96] (specialized to transporting iron into the cell), mannose,[97] heparin,[98] the transmembrane domain of diphteria toxin[99] and adenovirus.[100,101] Traditional poly-L-lysine-DNA complexes have been shown to localize to membrane-bound vesicles shortly after uptake and ultimately traffic to lysosomes, which represents a significant loss of delivery material. This problem can be avoided by conjugating the poly-L-lysine to adenoviruses and toxins, which are membrane-disruptive materials and capable of escaping the lysosomes.[101] However, the limitation of these approaches is the problem of immunocompatilibity, as these natural or synthetic haptens (small molecules which can elicit an immune response) with a linear polycation dramatically increases the immunoresponse and thus *in vivo* use might cause immunotoxicity and side effects.[102] Hence, the polymeric carrier development has been allocated to non-viral systems.

Several other polymeric carriers have been studied, such as polyethyleneimine,[103] poly-L-lysine without a viral carrier, dendrimers[104] and cyclodextrin[105]. Highly branched polymers are the most efficient delivery agents, though they are also the most toxic.[83] In most applications, one polymer (e.g. polyethyleneimine) has been grafted with another polymer (e.g. polyethyleneoxide) to maximize the

benefits of different polymers.[102] Often this is done to decrease interactions with blood constituents. Recently, research has focused on the applicability of cyclodextrins for ON transfer due to their unique ability to associate with other molecules. They have a hydrophilic outer surface and a hydrophobic central cavity, which is possible to derivatize with hydroxyl groups. The interaction properties of cyclodextrins with oligonucleotides are not as straightforward as those of spontaneously forming lipid complexes.[105] Problems with polymeric carriers have been similar to other systems: the best results have been achieved with the most toxic compounds.

Polymeric as well as inorganic nanoparticle formulations have been suggested for delivery purposes.[106,107] The most promising of the nanoparticle based transfection agents are the superparamagnetic nanoparticles, whose delivery to the target site can considerably be improved by an external magnetic field.[107]

Physical methods, such as electroporation[108], shockwaves[109] and ultrasound[110], may prove useful techniques for improved ON delivery. Electroporation is a delivery technique that utilizes high intensity electric fields to destabilize lipid bilayers. Shockwaves and ultrasound are techniques, where acoustic high-energy pressure pulses cause transient pore formation in cell membranes (cavitation).

Requirements for the delivery agents are strict. It seems that in many cases compromises are inevitable for the agents to be safe enough for patients. Thus, in most clinical trials none of the delivery agents are used due to lack of knowledge.[111,112]

One potentially safe group of delivery agents that has not been studied widely is a group of positively charged surfactants. These have been used for numerous medicinal purposes for many years and should not pose the same kind of safety risks as liposomes. In addition, surfactants are very membrane-active compounds. For these reasons, this work studies the use of surfactant compounds as delivery agents for oligonucleotides. Chapter 4 gives background information on surfactants and their interaction properties with polyionic and oligoionic compounds.

3.3. Antisense targets and clinical trials

Clinical trials in patients have recently begun to study the safety, patient tolerance and efficiency of antisense therapy. Most of the clinical trials with AONs are performed with the simplest forms of antisense ONs, typically phosphorothioates, without a carrier. Although some base-motifs discussed within this chapter have shown serious side effects in animal models, most AON drugs have generally been well tolerated. Dose-dependent side effects include thrombocytopenia, hypotension, asthenia and fever.[113]

The first antisense drug has already achieved US Food and Drug Administration (FDA) approval.[111,114] This AON drug is sold with a brand name, Vitravene®, and it is used against cytomegalovirus (CMV) retinitis in people with AIDS. It is applied directly to the eye through intravitreal injections and thus can function without a carrier. Many other clinical trials are on-going.[115]

The important focus of antisense therapy is on anti-cancer treatment.[116,117] The mode of action is mostly based on oligonucleotides, which decrease the expression of the oncoproteins (proteins that are coded for by a viral oncogene which has been integrated into the genome of a eukaryotic cell and that is involved in the regulation or synthesis of proteins linked to tumorigenic cell growth) or are focused on cell signaling molecules implicated in cancer initiation or progression.[118] Since Vitravene®, Genasense is the most studied and still very promising AON, whose target has been implicated in many cancers.[118] It is one of very few AONs, which have reached phase III in clinical trials. The issue, which might harm its success, is the CpG motif it contains.

Many other targets for AONs in anti-cancer treatment have also reached advanced phases in clinical development (melanoma, myeloma, lung cancer, leukemia).[119]As the phase I trials have concentrated on the safety of AON drug, the problem in phase II has been the efficiency of the drug either because the AON is not reaching the target tissue (delivery& stability problems) or it does not cause the effect, even if it does reach the target. It seems that these kinds of molecular treatments only tilt the balance between survival and death, and thus it is not probable that antisense therapy replaces traditional chemotherapy. [117] It may instead offer a synergistic way to improve the success of traditional treatments with novel gene technology.

At the moment, no medicinal treatments exist for the most common human genetic disease, polycystic kidney disease. AONs have given positive results in a first-phase clinical trial study and they have been proposed to replace dialysis or kidney transplantation treatments that are, at the moment, the only available treatments.[120] Viruses are also the target of AON treatments in phase I-III clinical trials (HIV, Hepatitis C/B, West Nile Virus).[121] Other on-going clinical trials for antisense drugs include the reduction of cholesterol and triglyserides in patients with high cholesterol as well as novel treatments for asthma and diabetes.[115,121]

Despite the few numbers of FDA approved AON drugs, there are rather recent success among other nucleic acid based drugs. Aptamers are molecules that bind to specific target molecules. Macugen® belongs to the class of nucleic acid based aptamers and it gained FDA approval in 2005.[122] This gives an indication that breakthroughs might already be close with other nucleic acid formulations. Only the future will reveal how many of the currently tested antisense drugs will proceed through clinical trials to commercial use.

4. Overview of surfactant-polyelectrolyte interaction

Surfactants are surface-active compounds consisting of two parts: a hydrophilic head and a hydrophobic tail. Due to their dual nature, they form numerous structures in aqueous solution. The most typical of these are micelles, where hydrophobic tails of the surfactants are packed together and hydrophilic head groups are oriented towards a bulk aqueous phase. Micelles are formed if the concentration of the solution exceeds a certain threshold value. This minimum is called the critical micelle concentration (CMC) and it is characteristic of a given surfactant. CMC depends on numerous factors such as the structure, charge and size of the head group, the length of the tail and the salt concentration of the solution.

Polyelectrolytes are macromolecules with covalently bound charged groups. Due to their high charge density, they have a tendency to carry a counter-ion cloud. This phenomenon is called counter-ion binding. The most well known counter-ion condensation theory was developed by Manning.[123] The theory approximates the polyelectrolyte chain as an infinite line charge with the charge density α. The charge is characterized with the charge density parameter, ξ defined as

$$\xi = \frac{e^2}{\varepsilon\varepsilon_0 kTb} \tag{10}$$

where e is the elementary charge, $\varepsilon\varepsilon_0$ is the permittivity of the surrounding (aqueous) solution, k is the Boltzmann constant, T is the absolute temperature and b is the distance between neighbouring charges. The permittivity of the solution is assumed to be uniform and equal to the bulk value. In the Manning theory, the charge density parameter ξ, describes the behaviour of the polyelectrolyte counter-ions. In the case of monovalent ions, the counter-ions condense on the polyelectrolyte if the initial value of ξ is more than 1. The condensation occurs until the parameter ξ decreases to unity. The theoretical explanation for the phenomenon is that the phase integral, which describes the energy in the system, is diverging when $\xi > 1$. Physically, this can be interpreted as the instability of such systems. When $\xi < 1$, the behaviour of the counter-ions can be treated with the Debye-Hückel theory[124]. This is the case for the uncondensed counter-ions as well as for polyelectrolytes having a low charge density.

In the following, the system of both a polyelectrolyte and surfactant of opposite charge in the aqueous solution will be considered. Oppositely charged surfactants are trapped in the electrostatic field surrounding the polyelectrolyte similarly to small counter-ions. This phenomenon was known already in the early 1900's, but was studied systematically for the first time in the 1980's.[125-128] Initially, it was assumed that the interaction between the surfactants and polyelectrolyte is purely electrostatic and the

surfactants are bound to the polyelectrolyte stoichiometrically with one surfactant attaching to one binding site. Later, it was discovered that the effect of hydrophobic interaction is strong, and the tendency of the surfactant tails to avoid water and bundle together is significant. Binding is believed to occur through the micellisation induced by a polyelectrolyte, which means that non-polar parts of the bound surfactants accumulate together and form aggregates, so called mini-micelles (Figure 6).[129] Alkyl chains of the surfactants are gathered to a hydrophobic core and their polar head groups are spread on the interface. Mini-micelles are considerably smaller than the conventional micelles of the corresponding surfactants. Some polyelectrolyte monomers interact with the polar head groups of the surfactants and hydrated alkylgroups, decreasing the free energy of the interface.[126] Other monomers form loops to the surrounding aqueous phase.

Binding is co-operative: after one surfactant is bound, the next one binds more easily due to hydrophobic interaction. The interaction between surfactants and polyelectrolyte is strong and it starts typically at concentrations that are orders of magnitude below the CMC of the corresponding surfactant. The surfactant concentration at the onset of binding is called the critical aggregation concentration (CAC).

Some properties of the polyelectrolytes strengthen the surfactant binding. If the polyelectrolyte is hydrophobically modified, the hydrophobic parts of the polyelectrolyte attract the surfactant tails and thus add to the attractive interaction between the surfactant and polyelectrolyte. In some cases, the attraction of the surfactant tails is so strong that surfactants and polyelectrolytes of the same charge also have a tendency to form complexes.[130-132]

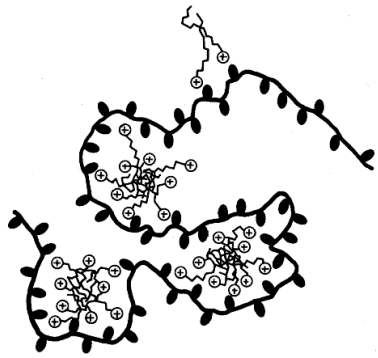

Figure 6. Schematic structure of a polyelectrolyte-surfactant complex. Reproduced with permission from [129] . Copyright 1984 American Chemical Society.

For most polyelectrolyte-surfactant systems, binding continues until the stoichiometric ratio of bound surfactants to charged groups is reached. This is often followed by precipitation of the neutralized complex.[133] Polyelectrolytes containing hydrophobic domains such as polystyrene sulphonate are most likely to precipitate.

4.1. Factors affecting surfactant-polyelectrolyte interaction

Many of the properties of a polyelectrolyte-surfactant solution are composition-dependent. Therefore the experimental techniques, which have been utilized for surfactant-polyelectrolyte research, have been numerous. The most common are potentiometric titration,[134], microcalorimetry[135] and techniques that are used to measure the surface tension,[136,137] conductivity,[138] light scattering,[139] NMR-spectra,[133] and fluorescence.[140]

The effect of the surfactant chemical composition on the interaction has been extensively studied. The surfactant alkyl chain length influences the polyelectrolyte-surfactant interaction in a similar manner as in the formation of micelles: the longer the chain length, the more favourable is the micelle/complex formation.[141] CAC decreases with increasing surfactant alkyl chain length, i.e. the strength of the interaction is strongest with long alkyl chain lengths. The head group size affects the binding such that increasing the size decreases the interaction forces between the polyelectrolyte and surfactants. This is due to the increased steric hindrance[140] as well as electrostatic screening[142]: bulky alkyl chains coil in the head group region and thus screen the charge of the surfactants. Cationic gemini surfactants are the latest group of interest.[143,144] They are dimeric surfactant molecules consisting of two hydrocarbon chains and two polar groups linked with a spacer. Interaction of the polyelectrolyte has been found to be much stronger with the gemini surfactant than with a single-chained surfactant and the interaction force is strongly dependent on the spacer length.[145]

Polyelectrolyte properties such as charge density, hydrophobicity and flexibility have a significant influence on the binding. With polyelectrolytes having a high charge density such as polystyrene sulphonates and oligodeoxynucleotides, the driving force for the association is primarily the electrostatic interaction and secondarily the hydrophobic interaction between the surfactant hydrocarbon tails. The lower the charge density of the polyelectrolyte, the more important is the hydrophobic effect of the surfactant tails. Hydrophobic domains of the polyelectrolytes participate in the binding process by interacting with the surfactant alkyl groups in the interfacial region. This is seen as lower CAC values compared to the case of a hydrophilic polyelectrolyte.[125] CAC is also highly dependent on the polyelectrolyte concentration.[146]

Although the size of the polyelectrolyte was previously thought to be irrelevant, it has now been shown that decreasing the size of the polymer increases CAC and decreases the co-operativity of binding, when the polymer size is below the threshold value.[134,147] For polyphosphates, this value is ca. 35 monomer units.[147] NMR studies have revealed that relatively rigid polyelectrolytes like polystyrene sulphonate form loose complexes with ionic surfactants, whereas flexible polyelectrolytes like polyacrylic acid form compact globules.[148]

The effect of temperature on the hydrodynamic radius of the surfactant polyelectrolyte complex has been studied and was found to be important.[139] Increasing the temperature as well as the salt concentration decrease the radius of the complex. The salt apparently screens the electrostatic interactions and also stabilizes the bound micelles in the complex.

4.2. DNA-surfactant interaction

If correctly chosen, cationic surfactants should be well suited for oligonucleotide delivery as they bind to poly- and oligoions already at very low concentrations, shield most of the negative charges of the oligonucleotide and should consequently make it more likely to be incorporated into a hydrophobic lipid membrane. Up to date, most of the research has been conducted with polyionic double-stranded DNA (dsDNA) strands. The surfactant types, for which interaction with dsDNA has been studied, include n-alkyltrimethyl ammonium[149-152] and alkanediyl-α,ω-bis-(dimethylalkylammonium[153,154]) salts. These surfactants prefer a micellar structure to a liposomal one in the aqueous phase. The interaction of cationic surfactants with dsDNA is very similar to their interaction with oppositely charged polyelectrolytes and can be described with the co-operative models discussed in the previous section. The structure of the complexes can be approximated as shown in Figure 6. However, dsDNA differs from traditional polyelectrolytes in many respects. dsDNA is more rigid and has a definite structure in the aqueous phase. This might affect the structure of the dsDNA-surfactant complex. Also, the base sequence has been shown to alter the structure.[155] Some published studies describe dsDNA-surfactant complexes as globular compact structures.[151,152] Dynamic light scattering studies have shown that the size of this globule is around 80 nm.[156] The experimental degree of binding of the surfactant was shown to be slightly less than the stoichiometric value[151,157] whereas some theoretical studies suggest that it is possible to invert the charge of dsDNA, if the surfactant is sufficiently hydrophobic.[158,159] This would be useful in dsDNA delivery since the carrier complex has to be able to penetrate the anionic cell membrane. Many of the above-mentioned properties of dsDNA-surfactant complexes are not valid for the less studied flexible oligonucleotides. In particular, the end effects have to be taken into account in the case of short oligoelectrolytes, which have reduced co-operativity to the binding of surfactants.[147]

Although there is no *in vivo* -data available for cellular delivery properties of dsDNA/oligonucleotide -surfactant complexes, the membrane activity of the dsDNA complexes has been modelled with biomembrane mimics. These include phospholipid vesicles[160], hydrophilic silicon oxide surfaces[161] and supported phospholipid films[162]. Naked dsDNA was shown not to adsorb at all to negatively charged or neutral cell membrane mimicking surfaces.[162] The adsorption was clearly observable when the cationic surfactant was present. The interaction was described as an incorporation of the dsDNA-surfactant complex into the hydrophobic interior of the membrane. For cellular delivery, this might not be sufficient, since the dsDNA/oligonucleotide has to be delivered to the cytoplasm in order to function as a drug. However, this might not prove to be problematic, as it has been proposed that negative lipids in the cytoplasmic wall of the cell membranes act as dsDNA/oligonucleotide releasers.[160]

5. Charge transfer in DNA

Publication V of this thesis considers the concept of lateral charge transfer in immobilized DNA layer on a silicon substrate.

Barton et al were the first to provide experimental proof of DNA-mediated charge transfer.[163] Using an assembly containing tethered metallointercalators, the group showed that when electrons are conducted along the DNA molecule, damage can appear up to 200 Å away from the site where the charge transfer was initiated.[164] Charge transfer through DNA from electron donor to electron acceptor is very sensitive to perturbations in base-pair structure, i.e., the DNA base-pair π-stack, which is formed when DNA adopts a double helical conformation. The charge is assumed to transfer entirely through the π-stack. It has been shown that charge transfer is inhibited by intervening DNA mismatches and bulges as well as by DNA-binding proteins that interfere with base pair stacking.[165-169] Other studies propose that charge transfers through the DNA double-strand over long distances using a hopping mechanism.[170,171] This mechanism proposes guanines (Gs) as carriers of the positive charge. According to these studies, the rate of the transfer is strongly dependent on the distances between individuals Gs. Electrical transfer has been shown to occur in short DNA molecules, in bundles and networks. Transfer is blocked in long single molecules that are attached to surfaces.[172]

The possibility to detect oxidative damage through long range DNA mediated charge transfer has been considered.[173] This technology has already been applied in the development of a biosensor which can detect single base mutations in DNA.[174] The device is based on a monolayer of thiol-terminated DNA duplexes assembled on a gold surface. A redox-active intercalator bound at the periphery of the DNA film is used to probe the DNA charge transfer. The reduction of the intercalator can be detected electrochemically when the DNA is fully Watson-Crick base-paired. If base-mismatches or other perturbations in the DNA π-stacking exist, the intercalator reduction will be attenuated. The sensitivity of the methodology can be further increased by a coulometric readout strategy with oxidation of the intercalator by ferricyanide present in the solution.[169] The intercalator is not catalytically active and cannot be oxidized, when the DNA contains a base-pair mismatch. Another example utilizing DNA mediated charge transfer for sensing applications includes the detection of protein binding to DNA containing binding sites.[168]

Although many hurdles remain, DNA mediated charge transfer offers a useful means of developing small and more sensitive biological probes for obtaining genomic information. Potential applications include the use of electrochemical methods to detect mismatches in DNA structure for pharmaceutical diagnostics, as well as the design of molecules that can carry out therapeutic DNA repair. Progress in this

area is dependent on further development in the fabrication of the electrode probes to useful arrays and the pre-treatment of the complex genomic DNA samples.[175]

Another field where charge transfer in DNA is receiving wide interest is in DNA nanoelectronics. This area of research makes use of the unique molecular recognition and structuring properties of DNA[176,177,178] to create novel, controllable electronic devices. To improve conductivity, the DNA sequence can be modified with metal ions or metallic nanoparticles[179]. Future applications envisioned use metal-doped DNA as nanoelectronic building blocks, in self-assembled hybrid nano-sized networks.

There has been an enormous amount of interest in the concept of charge transfer in DNA assemblies. In Publication V, the concept was probed with short fragments of DNA immobilised on silicon substrate. Particular interest was directed towards the effect of both the hybridized flat structures of Si-dsDNA and unorganized Si-ssDNA on the rate of the charge transfer.

6. Experimental setup and results

The aims of the experimental part of the thesis can be divided into two categories. In the first case, the aim is to study the potential of surfactants as oligonucleotide carrying agents. This includes studies of the stability of oligonucleotide–surfactant complexes, the kinetics of surfactant binding and the membrane activity of naked oligonucleotides and surfactant–oligonucleotide complexes. The results may help the oligonucleotide drug development to progress towards successful oligonucleotide cargo technology as well as extract important details of the surfactant–oligonucleotide interaction from the fundamental point of view. In the second case, the aim is to show the capability of modern electrochemical methods to extract information from the biological environment as well as to fine-tune these methods for solving complex biological problems.

6.1. Equilibrium studies

The first part of the experimental study considers the chemical equilibrium of the surfactants and oligoelectrolytes (short polyelectrolytes). Surfactant equilibrium and interaction properties (theory discussed in Chapter 4) were studied using both polystyrene sulphonates (PSS) of different lengths and oligonucleotides as the oligoelectrolyte compound. The purpose was to obtain information on the equilibrium conditions of the surfactant-oligoelectrolyte complexes and on the stability of the complexes.

The equilibrium studies were achieved using a micro-sized liquid-liquid interface introduced in Chapter 2. This interface configuration has previously been utilized for studying cation binding to DNA.[180] The liquid-liquid interface was in most cases formed at the end of the micropipette filled with organic solvent and dipped into an aqueous phase. In part of the studies, a microhole laser-drilled polymer membrane attached to the end of the measurement cell was used to support the liquid-liquid interface. In that case, the organic phase was immobilized with polyvinylchloride (PVC) as shown by Liljeroth *et al*.[49] The micropipette setup is shown in Figure 7.

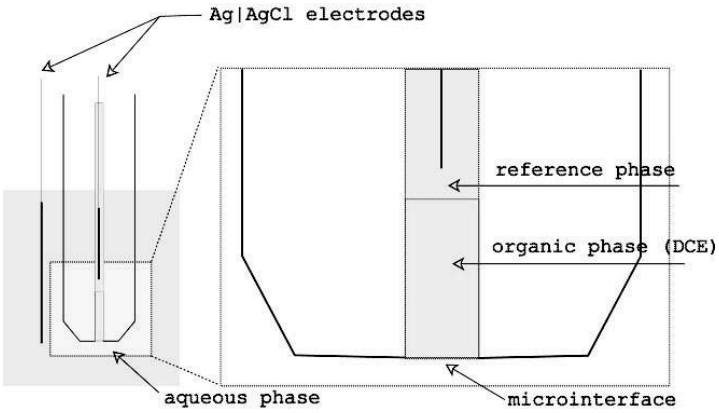

Figure 7. The experimental setup of the micropipette configuration.

The organic phase was either 1,2-dichloroethane (DCE) (micropipette) or 2-nitrophenyloctylether (NPOE) (microhole). The equilibrium concentrations of bound and free surfactants were obtained by an amperometric potential step method. The diffusion of the complex-bound surfactants was assumed to be negligible compared to the diffusion of free surfactants, which enabled the direct use of equation (6) for analyzing the steady-state current.

The results were analyzed using the model developed by Satake and Yang for estimating the degree of binding in co-operative binding reactions.[181]

$$\beta = 0.5\left(1 + \frac{Kwc_f - 1}{\sqrt{(1-Kwcf\,)^2 + 4Kc_f}}\right) \tag{11}$$

This model treats the polyelectrolyte as a linear array of binding sites and takes into account the co-operativity of the binding procedure. c_f is the concentration of the unbound surfactants. K is the intrinsic binding constant and is a function of the electrostatic interaction between the surfactant and the polymer. Kw can be calculated as following

$$c_f|_{\beta=0.5} = (Kw)^{-1} \tag{12}$$

where $c_f|_{\beta=0.5}$ is the free surfactant concentration when the degree of binding is 0.5. The slope of the binding isotherm determines the co-operativity parameter, w, which is a function of the hydrophobic interaction between adjacent bound surfactant molecules.

The surfactants studied in Publication I were cetylpyridinium chloride (CPC), dodecylpyridinium chloride (DPC), cetyltrimethylammonium bromide (CTAB), dodecyltrimethylammonium bromide (DoTAB) and tetradecyltrimethylammonium bromide (TTAB). These differ both in the length of the surfactant tail and the nature of the head group. The main conclusion from the differing tendencies of the mentioned surfactant configurations was that the binding affinity increases with increasing surfactant chain length. The result is in agreement with previous studies.[182]

The polyelectrolyte used in Publication I was polystyrene sulphonate (PSS) of three different chain lengths. The analyzed amperometric titration results are shown in Figure 8. The model described well the binding in the low binding degree region. The deviation from the theory was due to the phase separation near the saturation point of the polyelectrolyte. The co-operative equilibrium constant Kw, and co-operativity constant w, both increased as a function of polyelectrolyte size while the intrinsic equilibrium constant K, was essentially the same for all PSS, which shows that complexes of long polyelectrolytes are energetically more favourable than complexes of shorter oligoelectrolytes. This results from the more effective shielding of the hydrophobic polyelectrolyte-surfactant complex core from aqueous surroundings that is achieved with long polyelectrolytes. The behaviour of the shortest polyelectrolyte, only 9 monomer units long, differed essentially from others. The parameter w was lowest indicating an energetically less favourable structure for this complex. Possibly more than one polyelectrolyte chain took part in the complex aggregate formation, as suggested in previous studies.[183] The co-operativity of binding was decreased when the base electrolyte concentration was increased to 100 mM. In this case, the high base electrolyte concentration screens the short-range hydrophobic interaction between surfactant tails.

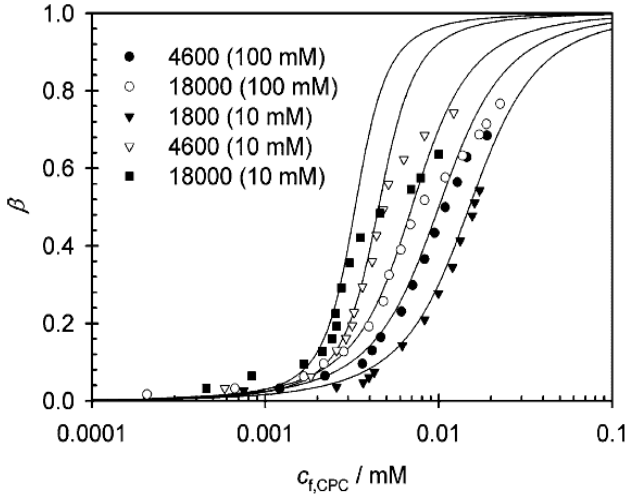

Figure 8. The experimental binding isotherms and the corresponding fitted Satake–Yang curves for three NaPSS samples of different chain lengths with molar masses 1800, 4600, and 18,000 g mol^{-1} of two different salt concentrations. Reprinted from Publication I, Copyright 2004, with permission from Elsevier.

The thermodynamics of binding was studied using microcalorimetry. The binding was shown to be an exothermic process with a favourable entropy change. The entropy change was lowest in the case of the 9 monomer long PSS, indicating that mini-micelle formation is not a significant factor in surfactant binding to short oligoelectrolytes.

In conclusion, Publication I showed that amperometric measurements using a microinterface as a detector interface can be used to directly measure the binding degree for surfactant binding to oligo- and polyelectrolytes. This is an improvement to earlier described potentiometric methods[125,128,141], as the introduced methodology enables the use of significantly smaller volumes and the measurements are quick and straightforward.

Publication II considered the surfactant-oligonucleotide binding equilibrium. CPC, already widely used in pharmaceutical applications such as mouthwashes, was used as the complexing agent. The effect of phosphorothioate modification (1st generation ON), in which one of the non-bridging oxygens of the sugar phosphate backbone of the oligonucleotide is replaced with sulphur, was studied. Two different base sequences were used, 5'-CCC CAT TCT AGC AGC CCG GG-3' (ODN1/MOD1) and 5'-GCC GAG

GTC CAT GTC GTA CGC-3' (ODN2/MOD2). ODN refers to phosphodiester and MOD refers to phosphorothioate.

The experimental normalized binding isotherms and corresponding fitted Satake–Yang curves are presented in Figure 9. The co-operativity equilibrium constant, *Kw*, was found to be significantly higher in the case of phosphorothioates, which shows that the CP–phosphorothioate complexes are energetically more favourable than complexes of phosphodiester and CP. This is explained by the modified charge distribution of the phosphate backbone due to different polarizability and the larger size of the sulphur atoms.

Base sequence had a surprisingly strong effect on binding. The co-operativity was affected most. The parameter *w* was clearly higher for ODN2 and MOD2 than ODN1 and MOD1, which is most probably due to the differing tendencies of oligonucleotides to form hairpin loop and dimer structures. Differences in *K* were insignificant.

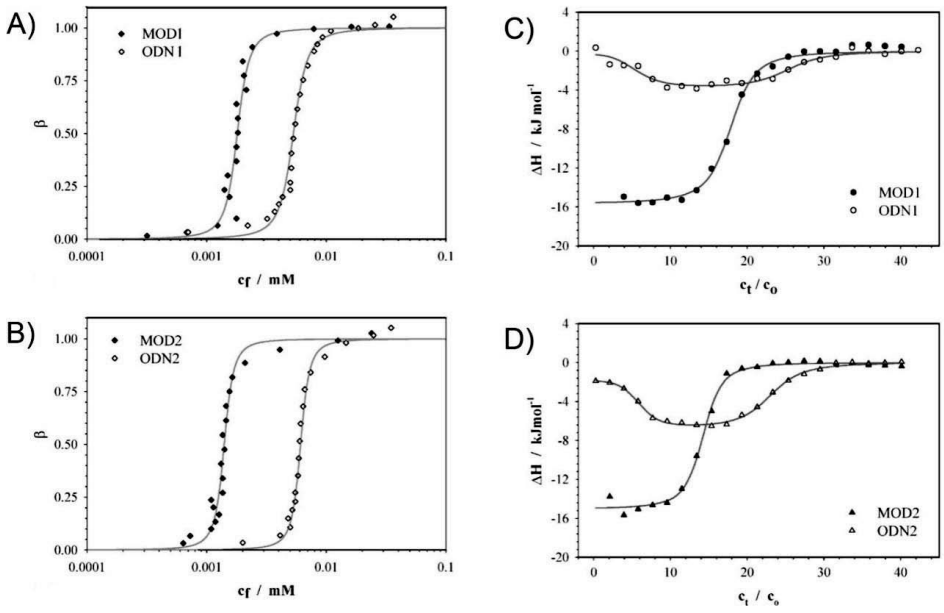

Figure 9. The experimental normalized binding isotherms and corresponding fitted Satake–Yang curves for (A) MOD1 and ODN1 and (B) MOD2 and ODN2. The enthalpy change in calorimetric measurements as a function of concentration ratio c_t/c_o for complexation of (C) MOD1, ODN1 and (D) MOD2, ODN2 at 25°C. Reprinted from Publication II, Copyright 2004, with permission from Elsevier.

Calorimetric studies were performed at a lower concentration range than the corresponding amperometric measurements due to endothermic CP micelle deformation reaction. The trends noted in the calorimetric studies were analogous to those already seen in the electrochemical studies: CP had a higher binding affinity to phosphorothioates than to natural oligonucleotides, which is evidenced by lower Gibbs free energies for phosphorothioates. Some base sequence specificity was also seen. The formation of phosphodiester oligonucleotide-CP complexes was clearly entropy driven, while enthalpy and entropy had an effect of the same order of magnitude on the Gibbs free energy in the case of phosphorothioate modifications.

In conclusion, Publication II considered the thermodynamics of surfactant binding to oligonucleotides. To the best of the author's knowledge, this was the first time that the binding of simple surfactant molecules to short ssDNA fragments was approached analytically. Earlier studies have either

been analytical studies of surfactant molecules binding with large dsDNA[149-154], or *in vitro* studies following the transfection efficiency of the DNA-surfactant complex.[184,185] In publication II, it was shown that the surfactant binding affinity and the surfactant-oligonucleotide complex stability differ for natural oligonucleotides and those with the phosphorothioate modification. This knowledge is essential in antisense oligonucleotide technology, since the stability of the complex has a significant influence on cellular delivery of oligonucleotides.

6.2. Complexation kinetics

Relatively few studies have considered the kinetics of the surfactant-polymer/polyelectrolyte interaction due mainly to the high reaction rate of the association reaction. The methods that have been used for studying this difficult phenomenon include relaxation techniques such as ultrasonic[186], temperature jump[187] and pressure jump methods[188]. Such methods are capable of studying rather slow dissociation of surfactants from the complex, but cannot follow a fast association process. In pharmaceutical applications, the kinetics of the interaction of two components is insignificant, provided it is sufficiently fast. However from a fundamental point of view, the subject is interesting. Thus in Publication III, hydrodynamic electrochemistry at ITIES is utilized for generating a system capable of following the kinetics of a fast homogenous reaction, such as surfactant binding to polyelectrolyte. The basics of hydrodynamic electrochemistry are presented in Chapter 2.

For measuring the kinetics of surfactant binding to polyelectrolyte, a channel flow arrangement was utilized, which enables control of the residence time of the oligoelectrolyte and surfactant by modifying the flow velocity in the channel. Different kinds of channel configurations have been proposed for combining two flow streams in a controlled manner, such as the flow injection channel and the confluence reactor.[189-191] In Publication III, the kinetics of the surfactant-polyelectrolyte interaction was studied with a new channel flow configuration, where surfactant solution was continuously injected into a flow of polyelectrolyte solution and the amount of un-associated surfactant was measured at an ion-selective detector-electrode. The configuration of the channel flow cell used in Publication III is shown in Figure 10.

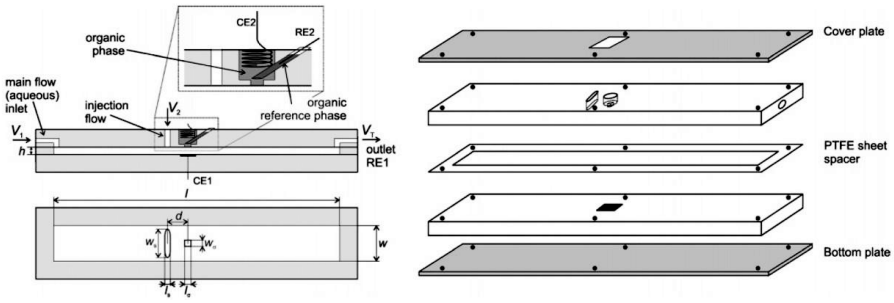

Figure 10. The flow cell configuration. CE1, CE2, RE1, and RE2 denote the aqueous and organic counter and reference electrodes, respectively. Reproduced with permission from Publication III. Copyright 2005 American Chemical Society.

The rate of surfactant ion transfer across ITIES (width, w_s) is controlled by the mass transfer in the channel and the rate of the homogenous association reaction. The association reaction could be modelled as a homogenous second-order reaction as the surfactant concentration was kept sufficiently low such that binding was limited only to the low binding degree region. In this region, the binding is perfectly non-co-operative. Two different approaches were used for modelling the second-order kinetics. Simple analytical theory assumed that flow rate only determines the residence time of the polyelectrolyte and surfactant. Another approach was a finite-element simulation of the full convective diffusion equation, which included the homogeneous chemical reaction.

The analytical solution overestimated kinetic parameters due to the approximations made in the residence time evaluation. However, the equilibrium constants determined were similar in the analytical and numerical finite-element solutions and of the same order of magnitude as the intrinsic equilibrium constant of CP binding to PSS describing the equilibrium in a non-co-operative system (Publication I).

The simple reversible second-order theory explained the results surprisingly well. This can be rationalized by the low concentration of the surfactant used. In this case, only few surfactant molecules associate with the empty binding sites of the polyelectrolyte and the co-operativity, which is a typical property of the polyelectrolyte-surfactant system, was assumed to have only a minor effect on the association of CP and PSS. The experimental results and fits to the analytical model are shown in Figure 11.

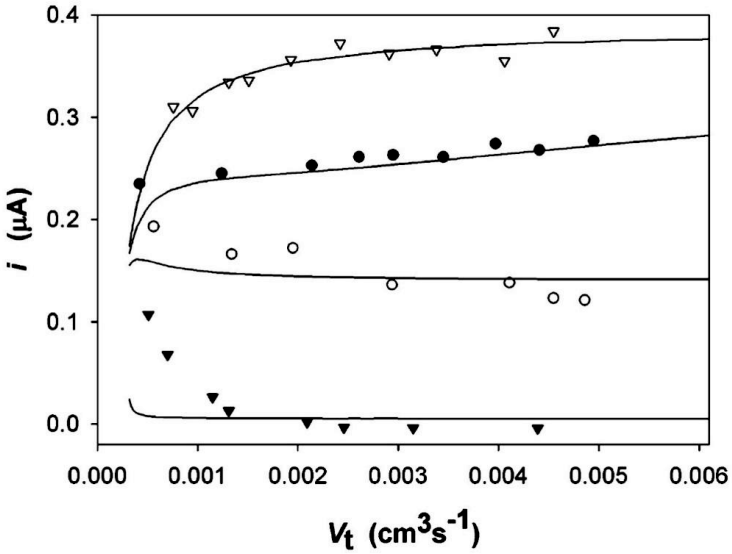

Figure 11. Experimental results and fits to the analytical model. Empty triangles, filled circles, empty circles, and filled triangles refer to PSS0, PSS5, PSS10, and PSS97, respectively. Solid lines refer to the reversible model discussed in the text. Reproduced with permission from Publication III. Copyright 2005 American Chemical Society.

In conclusion, Publication III introduced a channel flow method where a liquid-liquid interface was used as a detector electrode. This kind of electrode was described earlier by Liljeroth *et al.*[192] In Publication III, the detector electrode was combined with a novel channel flow configuration, which enabled controllable injection from the smaller side stream into the mainstream and thus the following of the complexation reaction of the polyelectrolyte and surface active agent for the first time. The configuration of the channel was a modification of that introduced by Gooding *et al.*[189,190]

6.3. Membrane activity of oligonucleotide-surfactant complex

Study of the incorporation of the oligonucleotide-surfactant complex into a lipid membrane is a natural extension of the previous studies considering the properties of the oligonucleotide-surfactant complex. The work presented in Publication IV utilizes electrochemistry at ITIES for studying the interactive properties of oligonucleotides and a natural cell membrane mimicking the phospholipid monolayer. The overview of development of the electrochemical techniques for studying ion transfer processes through biomembrane mimicking phospholipid interfaces was presented in Chapter 2.

In previous works, the membrane activity of surfactant-oligonucleotide complexes have been studied using silicon oxide surfaces,[161] phospholipid vesicles[160] and supported phospholipid films.[162] The approach used in Publication IV was to employ a phospholipid monolayer supported on the surface of the immobilized organic gel and follow the membrane interaction of the oligonucleotide and surfactant by voltammetric methods. This approach has been successfully utilized for studying the membrane activity of peptides.[193]

The phospholipid monolayer was prepared using the Langmuir-Blodgett technique, which enables control of the lipid packing at the air-water interface by movable barriers. The electrochemical cell (Figure 12) was dipped through the monolayer compressed to a surface pressure at which the lipid packing is in the liquid-condensed state. The substances under study were added to aqueous phase and their membrane activity was followed by cyclic and AC-voltammetry.

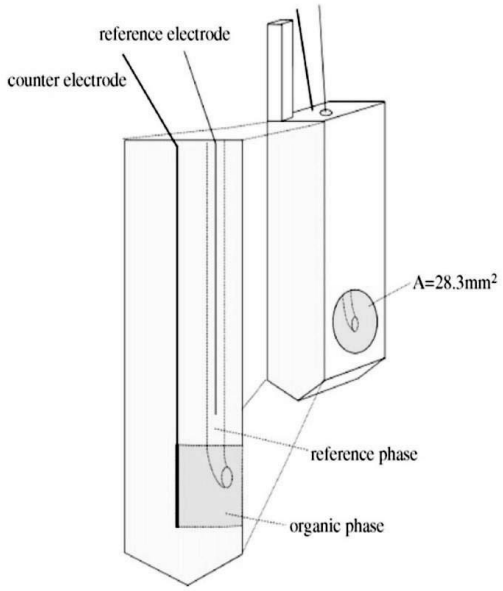

Figure 12. A schematic drawing of the electrochemical cell. Reprinted from Publication IV, Copyright 2006, with permission from Elsevier.

The capacitance of the monolayer as a function of $\Delta_o^w \phi$ was extracted from the AC-voltammetry results. With the aid of theoretical modeling based on the solution of the Poisson-Boltzmann equation, the capacitance values give information on the interfacial adsorption of the oligonucleotides and their surfactant complexes.

The molecules used in this study were the same as those used in Publication II: CPC, ODN1/2 and MOD1/2. The lipids used were zwitterionic 1,2-dipalmitoyl-sn-glycero-3-phosphocholine and negatively charged 1,2-dipalmitoyl-sn-glycero-3-[phospho-L-serine] (sodium salt).

The experimental capacitance curves are shown in Figure 13. The oligonucleotide proved not to interact with the neutral or negatively charged lipid membrane. When oligonucleotides were complexed with the surfactant, the membrane activity of oligonucleotide clearly increased, as concluded from the fact the capacitance increased and the surface charge decreased as a function of complexation degree. It was concluded that the complexes were incorporated to the phospholipid domain. The internalization did not require reversal of the negative charge of the oligonucleotide. However, complexed MOD1 did not adsorb to the lipid domain and it was assumed that MOD1 preferred stable complexes with the surfactant over internalization as the equilibrium constant *K* is highest for MOD1 (shown in Publication II).

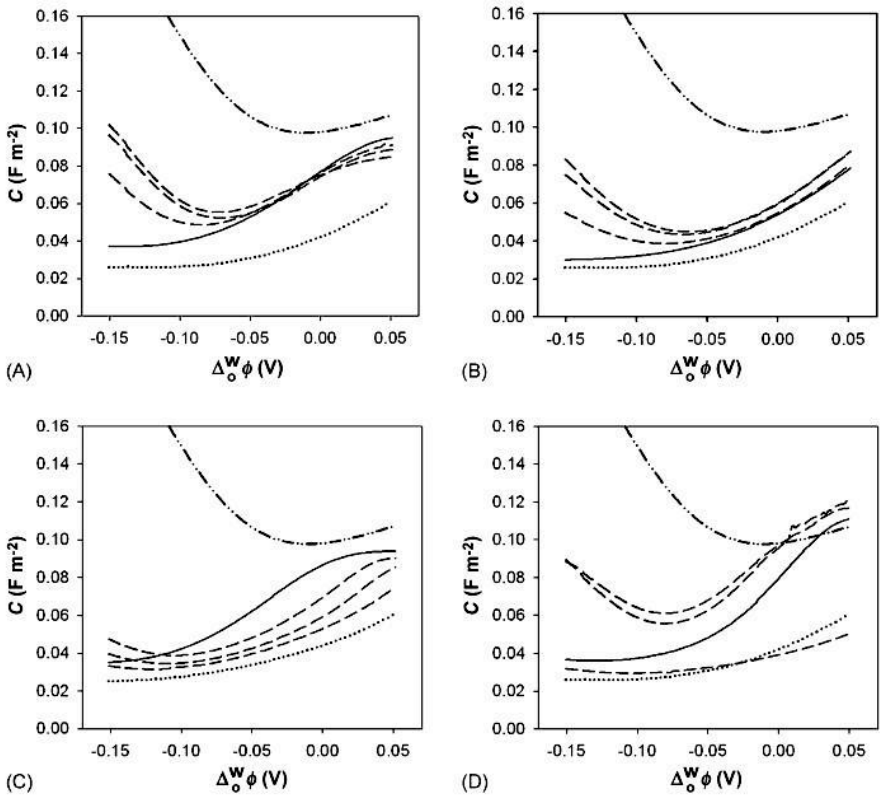

Figure 13. Experimental capacitance curves: (A) ODN1. The solid lines represent data obtained in the presence of naked oligonucleotide and the dashed lines represent those obtained in the presence of the complexed oligonucleotides with the CP/oligonucleotide concentration ratios of 0.24, 0.71 and 0.96 from bottom to top. The dotted lines represent the response for the compressed lipid monolayer with only base electrolytes in the aqueous compartment and the dash-dot-dot line shows the bare interface without lipid monolayer, (B) ODN2, (C) MOD1 and (D) MOD2. The lines are assigned as in (A). Reprinted from Publication IV, Copyright 2006, with permission from Elsevier.

In conclusion, publication IV utilized the Langmuir-Blodgett method in combination with modern electrochemical techniques to study the membrane activity of the oligonucleotides. The technique used was earlier presented by Liljeroth *et al.*[49] For the first time, differences in the lipid monolayer internalization of naked and complexed ONs and their phosphorothioate modifications were studied systematically using analytical methodology. The results show that cationic surfactants enhance the

internalization of the oligonucleotides into the phospholipid membrane with possibly a very similar mechanism to that shown for oligonucleotide delivery via liposomes.[90] However, results do not show whether the oligonucleotides can escape from the lipid vesicles, which has been a problem with many delivery systems. Nonetheless, this may not be an issue with a surfactant-based delivery system, since the inner cell surface is rich with negative lipids, which associate with positive surfactants and possibly act as oligonucleotide releasers.[90]

6.4. Lateral Charge in DNA-monolayer immobilized on the Si(111) electrode

Surface-bound DNA is important in analytical applications and nanotechnology, as it is a potential future material for constructing nanoarchitectures. Knowledge of the charge transport in the DNA-monolayers is essential, for the immobilized DNA monolayers to be used as components in sensor applications. In Publication V, SECM was used to verify the possibility of charge transport in the DNA monolayers, which are covalently bound to a Si(111) surface. The protocol shown in Figure 14 was used in the synthesis of DNA on the silicon surface.

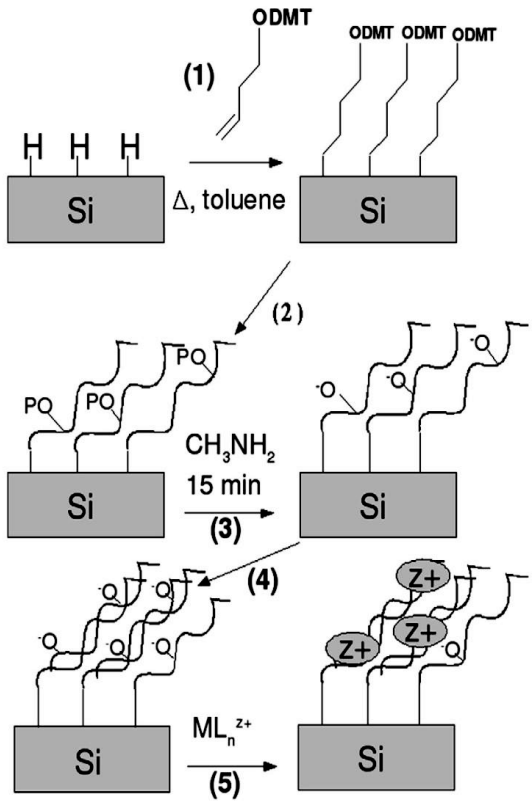

Figure 14. Synthesis of oligonucleotides on silicon surfaces. (1) Alkylation of hydrogen terminated silicon with 4,4'-dimethoxytrityl-1-undecenol [DMT = dimethoxytrityl]. (2) Solid-phase oligonucleotide synthesis resulting in 3'-tethered, protected ssDNA strands. P = $NC(CH_2)_2$. (3) Deprotection of the ssDNA with gaseous dimethylamine to remove the protecting groups including the cyanoethyl groups on the phosphate backbone. (4) Hybridisation with the complementary strand to form dsDNA. (5) Equilibration of the surface-bound DNA with metal complex. Reprinted from Publication V, Copyright 2007, with permission from Elsevier.

SECM studies were performed in amperometric mode, where the current response of the tip is measured as a function of the distance between the tip and the silicon surface (see Figure 15). The corresponding plots of tip current versus tip distance from the substrate, so-called approach curves provide quantitative information on the conductivity of the substrate.

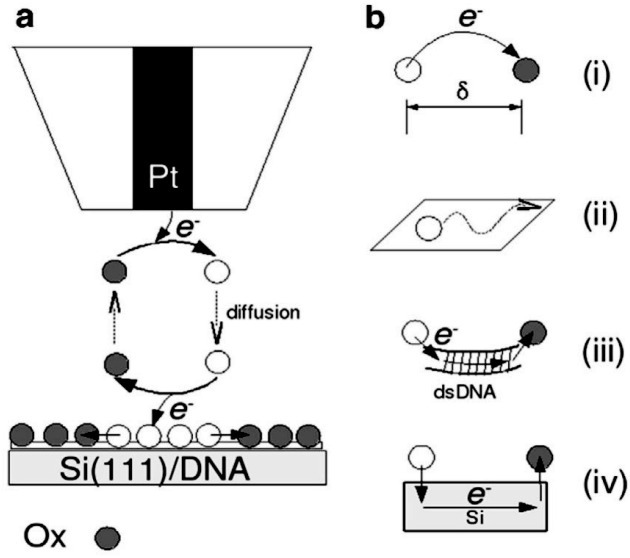

Figure 15. Illustration of the SECM experiment to determine surface charge transport rates between metal complexes bound to DNA. The tip reaction perturbs the equilibrium between freely diffusing and surface bound mediator leading to a flux of charge/material across the surface. (b) Different possible interpretations of the apparent surface diffusion coefficient as measured by the SECM experiment (a): (i) redox hopping between fixed centres, (ii) physical diffusion, (iii) long-range electron transfer mediated by dsDNA and (iv) injection of electrons or holes into the underlying silicon leading to positive feedback. Reprinted from Publication V, Copyright 2007, with permission from Elsevier.

The reaction at the SECM tip is

$$O_{soln} + e^- \rightarrow R_{soln} \quad (13)$$

The depletion of oxidized mediator (O) and accumulation of reduced mediator (R) in the tip/substrate gap favours the reduction of surface-bound molecules.

$$R_{soln} + O_{surface} \rightarrow O_{soln} + R_{surface} \quad (14)$$

The apparent surface diffusion coefficient, D_{surf}, for the lateral charge transport was determined by fitting experimental current vs. distance curves to an appropriate model. It is known that electron transport

by hopping between redox sites follows Fick's law, and in this way, it is equivalent to physical diffusion of molecules.[194, 195] The values of D_{surf} extracted from the SECM experiment were therefore interpreted in terms of the different possible underlying mechanisms of charge transport (Figure 15): (i) electron hopping between fixed, neighbouring redox sites; (ii) physical diffusion, in which the molecules are mobile on the surface or may detach and re-adsorb following diffusion in the solution; (iii) long-range electron transfer between well-separated sites via injection of charge into the DNA molecules; and (iv) charge injection into the underlying semiconductor substrate.

SECM approach curves were measured for $Fe(CN)_6^{4-}$, $Ir(Cl)_6^{3-}$, $Ru(bipy)_3^{2+}$, $Co(bipy)_3^{3+}$ and $Ru(NH_3)_6^{3+}$ (hexacyanoferrate(II) anion, hexachloroiridate(III) anion, Tris(bipyridine)ruthenium(II) cation, Tris(bipyridine)cobalt(III)) cation and hexaammineruthenium(III) cation) at Si(111)/dsDNA surfaces. The two anionic redox mediators produced only negative feedback. This is attributed to the Donnan exclusion of the negatively charged species from the polyanionic Si/DNA film. No measurable feedback was observed for $Ir(Cl)_6^{3-}$ at Si(111)/dsDNA surfaces, although it has an increased redox potential. In contrast to $Ir(Cl)_6^{3-}$, a significant positive feedback was observed using $Ru(bipy)_3^{2+}$ as mediator. This effect was observed independently of the presence of DNA on the surface, i.e., also on hydrogen-terminated and undecyl-monolayer covered Si electrode, which shows that in the system any DNA oxidation effect was obscured by hole injection into the valence band of the underlying Si substrate. It was concluded that the standard redox potential of $Ru(bipy)_3^{2+}$is sufficiently positive to invert the surface locally as positive feedback was also observed at n-Si substrates.

When the approach curve measurement was performed in the presence of $Ru(NH_3)_6^{3+}$, it was noted that DNA molecules on top of the organic monolayer have a clear effect on the approach curve increasing the positive feedback response. The extent of positive feedback increased after hybridisation. In the absence of DNA, the approach curves were indistinguishable from those obtained at insulating substrates. This indicated that the regeneration of the mediator is slow at the underlying Si surface. The positive feedback observed must therefore be at least partly due to lateral charge transport in the Si/DNA film. Figure 16 shows feedback approach curves for 0.50 mM $Ru(NH_3)_6^{3+}$ at pSi/DNA surfaces.

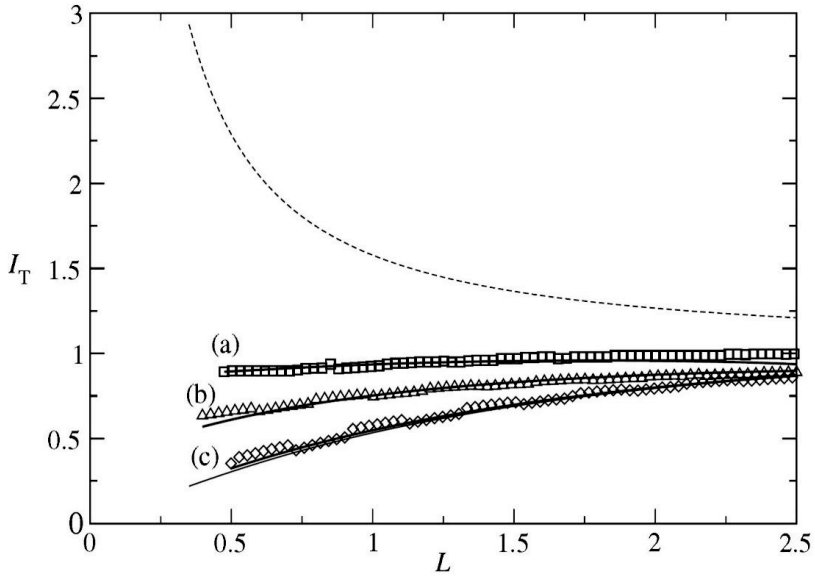

Figure 16. Feedback approach curves for 0.50 mM $Ru(NH_3)_6^{3+}$ at pSi/DNA surfaces. The electrolyte was 20 mM Tris buffer at pH 7.5 and the tip radius was 5 µm. The tip potential was -0.3 V vs. SCE. The symbols are the experimental data and the lines are the fitted curves. (a) pSi/dsDNA, (b) pSi/ssDNA and (c) pSi/PG-DNA. dsDNA and ssDNA indicate hybridised and single-stranded DNA. PG-DNA indicates single stranded material prior to deblocking of the phosphates and nucleobase protecting groups. The theoretical responses for diffusion-controlled positive feedback (metallic substrate) and pure negative feedback (insulating substrate) are also shown as dashed and thin solid lines. Reprinted from Publication V, Copyright 2007, with permission from Elsevier.

In previous studies, various electroanalytical techniques have been employed to detect hybridization of DNA immobilized on electrode surfaces.[196,197] In Publication V, charge transfer on the DNA covalently bound on silicon surface was studied using SECM methodology. A semi-analytical description of the SECM feedback experiment with lateral charge transport on the substrate was developed and used to analyse steady-state approach curves in solutions of various metal complex mediators at Si(111)/DNA substrates. The mechanism of this process was elucidated for DNA films equilibrated with different metal ion complexes. The inverse dependence of the effective first-order heterogeneous rate constant at the substrate on bulk mediator concentration predicted by theory was confirmed using the p-Si(111)/dsDNA/ $Ru(NH_3)_6^{3+}$ system. $Ru(NH_3)_6^{3+}$ bound to surface-immobilised ssDNA molecules was found to transport charge across the surface with an apparent diffusion coefficient of $(0.85 \pm 0.3) \cdot 10^{-5}$ cm^2 s^{-1}, which is

similar to the diffusion coefficient value in solution. The rate of charge transport increased after hybridisation to $(2.2 \pm 0.3) \cdot 10^{-5}$ cm^2 s^{-1}. This high apparent diffusion coefficient is interpreted in terms of a combination of physical diffusion of the metal complexes on the surface and electron injection into the underlying semiconductor. In the Si/dsDNA system, the DNA strands lie almost parallel to the surface and therefore lateral charge transport can be facilitated. In the case of Si/ssDNA, there is little structure in the films as shown by AFM (Figure 17) and the apparent diffusion coefficient was comparable to the value in the bulk solution.

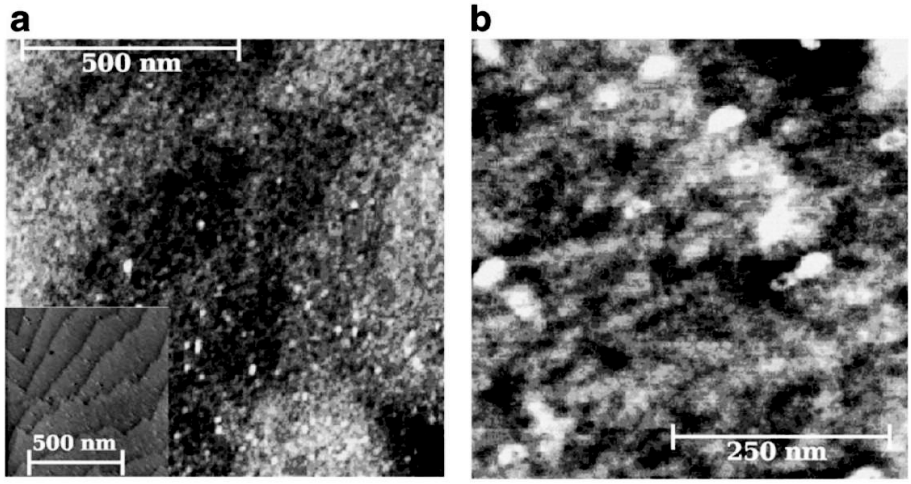

Figure 17. AFM images of ssDNA-PG (before) and ssDNA after deblocking with methylamine (Fig. 14). (a) 1 · 1 µm tapping mode AFM image of ssDNA-PG; the underlying step/terrace structure of the Si(111) surface is visible on the right-hand side. The grayscale for the AFM image is 0–2 nm. The inset shows an in-air STM image at -2 V bias and 0.3 nA tunnelling current of the Si(111)–C11–OH monolayer showing steps (0.3 nm) equal to the monoatomic step height on Si(111)–H: this indicates the monolayer covers the surface uniformly. (b) 500 · 500 nm tapping mode image of the ssDNA surface after deblocking the cyanoethyl protecting groups on the phosphate backbone with methylamine. The grayscale for the AFM image is 0–1 nm. Reprinted from Publication V, Copyright 2007, with permission from Elsevier.

7. Conclusions

There were two major aims for this work. The first one was to showcase the versatility of electrochemical techniques for studying biological problems. The second was the utilization of electrochemistry for studying surfactant-mediated anti-sense oligonucleotide delivery. These goals were fulfilled successfully and new information was extracted on biological systems relevant to the pharmaceutical community. In the first part of this work, electrochemical techniques at micro-scale and hydrodynamic liquid-liquid interfaces were utilized for studying the thermodynamic stability and kinetics of complex formation for surfactants and oligonucleotides and their model compounds. The capability of the micropipette method to study the complex formation was proved using polystyrene sulphonate as a model compound. The experimental results were fitted successfully to Satake-Yang model and the results were compared with the relevant literature. The results with oligonucleotides were promising as they showed that oligonucleotides and cetylpyridinium cations form stable complexes with binding degrees close to unity. Thus, the charge of the oligonucleotide is effectively shielded in these complexes. Formation of the complexes is thermodynamically favourable in aqueous environments. Binding of cetylpyridinium to oligonucleotide starts at much lower cetylpyridinium ion concentration in the case of phosphorothioate based oligonucleotides compared with the unmodified phosphodiesters. Binding is entropy driven in the case of phosphodiesters while enthalpy and entropy induce effects of same order of magnitude in the case of phosphorothioates. The base sequence of the oligonucleotide has a clear effect on the binding thermodynamics, most probably due to the different tendencies of oligonucleotides to form loop, hairpin and other secondary structures. The kinetics of surfactant binding to the oligonucleotide were studied as a homogenous second order reaction, which was possible, as the concentration of the surfactant was kept sufficiently low for binding to be limited to the non-co-operative low binding degree region. The results were analyzed with the analytical theory and simple second-order model. The simple theory explained the results surprisingly well due to low surfactant concentration that was used. The membrane activity of the complexes was studied using an artificial membrane built by a Langmuir-Blodgett technique combined with electrochemical methods. The results were analyzed using the solution of the Poisson-Boltzmann equation. It was concluded that the surfactant-oligonucleotide complexes incorporated into the lipid membrane with the exception of one oligonucleotide-surfactant complex with the phoshorothioate backbone. The stability of the complex was concluded to be a critical factor affecting the complex – phospholipid membrane interaction: the more stable the complex, the more difficult is its incorporation into the phospholipid region. The complexes that penetrated the membrane did not require absolute shielding of the negative charges of the oligonucleotides. Knowledge of the charge transport in the DNA monolayers is essential, when immobilized DNA monolayes are used as components in sensor applications or electronic devices. SECM was used to verify the possibility of charge transfer in DNA

monolayers, which are covalently bound to a Si(111) surface. It was concluded from the positive SECM feedback that the charge transfers in the dsDNA monolayer laterally. As AFM images showed, the ssDNA films on silicon have a random structure and the charge transfer rate was comparable to the value of the bulk solution. The results with dsDNA are very promising for future sensor technology. As a whole, this work showed that with the modern electrochemical techniques it is possible to take a close analytical perspective of the complex biological problems.

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